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THE HYPERCALCEMIC ACTIVITY OF DIHYDROTACHYSTEROL$_2$ AND DIHYDROTACHYSTEROL$_3$ AND OF THE VITAMINS D$_2$ AND D$_3$

COMPARATIVE EXPERIMENTS ON RATS

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(*Received* 21 *December* 1958)

Abstract—The hypercalcemic activities of dihydrotachysterol$_2$, dihydrotachysterol$_3$, vitamin D$_2$ and vitamin D$_3$, have been compared at different intervals (2, 4, 7 and in some cases 1 and 10 days) after the administration to rats of one oral dose of the crystalline compounds in pure peanut oil. The maximal serum calcium levels appear to be obtained 2–4 days after the administration. From the results, it appears that dihydrotachysterol$_3$ is the most active hypercalcemic agent, followed, in decreasing order, by dihydrotachysterol$_2$, vitamin D$_3$ and vitamin D$_2$.

The activity ratios proved to be highly dependent on the time interval. As to the hypercalcemic activity, the potency ratio vitamin D$_3$/vitamin D$_2$ has been found to be about 1·6, while, with respect to their antirachitic activities, both vitamins are equipotent in rats. The antirachitic activity of dihydrotachysterol$_3$ was shown to be nearly twice as high as the potency of dihydrotachysterol$_2$, the antirachitic activity of dihydrotachysterol$_2$ being about 0·5 per cent of the vitamin D$_3$ activity.

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INTRODUCTION

In addition to their antirachitic activities the vitamins D also raise the level of serum calcium after being administered in relatively large quantity.

However, dihydrotachysterol$_2$,[5, 8, 9, 12] a compound which is structurally closely related to vitamin D$_2$, is more specific in this respect. This agent has been in clinical use for a long time already in cases of hypoparathyroidism, but the opinions differ concerning the respective activities of vitamin D$_2$, vitamin D$_3$ and dihydrotachysterol$_2$ and also with regard to their mode of administration.[4, 6, 7]

After dihydrotachysterol$_3$ (structurally closely related to vitamin D$_3$) was synthesized,[11, 13] it seemed worth while to start experiments with rats in order to compare quantitatively the hypercalcemic effects of the two dihydrotachysterols and the two vitamins D after one oral administration in oily solution.

For these experiments, we had these four compounds in a pure, crystalline form at our disposal.

The serum calcium levels have been determined 2, 4 and 7 days (and in some experiments also 1 and 10 days) after administering the compounds.

EXPERIMENTAL PART

For the determination of the hypercalcemic activity we chose the method of MacChesney and Kocher,[1] which was modified in the following way:

For each single experiment 98 male rats (average body weight ± 200 g) of our own

inbred stock were prepared by feeding a stock diet, to which 1 per cent calcium carbonate was added, for at least 10 days.

These animals were divided into 7 uniform groups of 14 rats each: 6 for the 2 substances to be compared (each substance was tested in 3 doses) and one control group.

The drugs in peanut oil (0·5 ml) were given to fasting animals by means of a stomach tube in 0·5 ml volumes. The control rats received in the same way 0·5 ml pure peanut oil.

The quantities, administered per rat were:

dihydrotachysterol$_2$: 0·25, 1·0 and 4·0 mg

dihydrotachysterol$_3$: 0·1, 0·4 and 1·6 mg

vitamin D_2 and D_3: 0·25, 1·0 and 4·0 mg and 1·0, 4·0 and 16·0 mg.

After the fixed intervals (1, 2, 4, 7 or 10 days), the rats were anaesthetized and their blood collected individually. The blood samples were centrifuged and the sera mixed in pairs (equal volumes) within the groups. In these mixed sera the calcium determinations (7 in each group) were performed according to the method of Clark and Collip.[8]

Each experiment (98 rats) was executed twice (experiments I and II), at different moments, sometimes several months apart. The data were worked up statistically. Afterwards, the results of each pair of experiments (experiments I and II) were combined statistically.

RESULTS AND DISCUSSION

In Tables 1A, 1B and 1C, the data of the 24 single experiments are collected, respectively for the following comparisons:

dihydrotachysterol$_3$/dihydrotachysterol$_2$,

dihydrotachysterol$_2$/vitamin D_3 and

vitamin D_3/vitamin D_2.

In all cases, the serum calcium values are given as increases over the corresponding control values. Also the standard deviations of the mean values are given and, in parentheses, the numbers of determinations made. With the higher doses, toxicity caused loss of animals. Therefore, the number of determinations quoted in the tables are sometimes less than seven.

The maximum increases in serum calcium appear about 2–4 days after ingestion of the drug. From the serum calcium values, the activity ratios have been calculated according to the method mentioned in U.S.P. XV.[10] One of the compounds under investigation is considered as a standard preparation and the activity ratio indicates the biological activity of the second compound ("test preparation") in relation to the biological activity of the standard. The results are summarized in Table 2 for the experiments I and II. The weighed mean values, with their P 0·95 intervals, are also given.

The results calculated from the serum calcium values 1 and 10 days after administration are given between parentheses. As can be seen from Tables 1A and 1C, the increases in serum calcium fail to show a sufficient dose–response relation resulting in the absence of a reliable slope of the curve. In these experiments, vitamin D_2 has been put forward as the weakest substance in hypercalcemic activity. It is followed, in increasing order, by vitamin D_3, dihydrotachysterol$_2$ and dihydrotachysterol$_3$. In Table 3 we have put the hypercalcemic activity of dihydrotachysterol$_2$ equal to one for each interval (2, 4 and 7 days), and recalculated the activity relations.

TABLE 1A. COMPARISON OF DIHYDROTACHYSTEROL$_3$ WITH DIHYDROTACHYSTEROL$_2$. MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg per cent)

Days after administering	Dihydrotachysterol$_3$			Dihydrotachysterol$_2$		
	0·1 mg	0·4 mg	1·6 mg	0·25 mg	1·0 mg	4·0 mg
1 (Expt. I)	1·36 ± 0·25 (7)	1·59 ± 0·35 (7)	1·11 ± 0·26 (7)	1·15 ± 0·17 (7)	1·82 ± 0·23 (7)	2·06 ± 0·20 (7)
1 (Expt. II)	0·86 ± 0·14 (7)	0·91 ± 0·16 (7)	1·02 ± 0·24 (7)	0·61 ± 0·14 (7)	0·90 ± 0·13 (7)	1·21 ± 0·15 (7)
2 (Expt. I)	2·78 ± 0·19 (7)	3·92 ± 0·58 (7)	5·55 ± 0·22 (7)	1·83 ± 0·21 (7)	3·87 ± 0·34 (7)	5·40 ± 0·30 (7)
2 (Expt. II)	1·88 ± 0·18 (7)	3·56 ± 0·25 (7)	5·37 ± 0·04 (3)	1·45 ± 0·18 (7)	3·01 ± 0·26 (7)	4·90 ± 0·16 (7)
4 (Expt. I)	3·25 ± 0·20 (7)	4·76 ± 0·28 (7)	5·04 ± 0·32 (5)	1·58 ± 0·22 (7)	3·83 ± 0·22 (7)	4·77 ± 0·17 (5)
4 (Expt. II)	3·18 ± 0·13 (7)	4·37 ± 0·34 (6)	5·81 ± 0·25 (6)	1·96 ± 0·13 (7)	3·93 ± 0·17 (7)	5·03 ± 0·20 (7)
7 (Expt. I)	1·38 ± 0·21 (7)	2·61 ± 0·32 (5)	4·31 ± 0·56 (3)	0·95 ± 0·16 (6)	2·21 ± 0·27 (7)	3·21 ± 0·28 (6)
7 (Expt. II)	1·01 ± 0·16 (7)	2·96 ± 0·22 (7)	3·71 ± 0·23 (2)	0·80 ± 0·10 (7)	2·47 ± 0·27 (7)	4·29 ± 0·56 (3)

TABLE 1B. COMPARISON OF DIHYDROTACHYSTEROL$_2$ WITH VITAMIN D$_3$. MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg per cent)

Days after administering	Dihydrotachysterol$_2$			Vitamin D$_3$		
	0·25 mg	1·0 mg	4·0 mg	0·25 mg	1·0 mg	4·0 mg
2 (Expt. I)	2·44 ± 0·32 (7)	3·89 ± 0·24 (7)	4·69 ± 0·31 (7)	0·81 ± 0·06 (7)	1·56 ± 0·19 (7)	3·41 ± 0·29 (7)
2 (Expt. II)	0·94 ± 0·15 (7)	3·48 ± 0·33 (7)	3·64 ± 0·38 (6)	0·77 ± 0·12 (7)	0·91 ± 0·22 (7)	3·91 ± 0.41 (6)
4 (Expt. I)	1·42 ± 0·15 (7)	3·38 ± 0·17 (7)	3·99 ± 0·21 (5)	0·72 ± 0·17 (7)	1·99 ± 0·19 (7)	3·80 ± 0·32 (6)
4 (Expt. II)	1·56 ± 0·30 (7)	3·23 ± 0·28 (6)	4·04 ± 0·34 (7)	0·47 ± 0·28 (7)	1·91 ± 0·15 (7)	3·28 ± 0·24 (6)
7 (Expt. I)	0·79 ± 0·14 (7)	2·78 ± 0·31 (7)	4·09 ± 0·17 (5)	0 27 ± 0·08 (7)	1·09 ± 0·16 (7)	2·41 ± 0·17 (7)
7 (Expt. II)	1·31 ± 0·36 (7)	2·51 ± 0·42 (6)	3·42 ± 0·46 (5)	0·71 ± 0·18 (7)	1·37 ± 0·14 (7)	3·38 ± 0·22 (5)

TABLE 1C. COMPARISON OF VITAMIN D_3 WITH VITAMIN D_2. MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg per cent)

Days after administering	Vitamin D_3			Vitamin D_2		
	1·0 mg	4·0 mg	16·0 mg	1·0 mg	4·0 mg	16·0 mg
1 (Expt. I)	1·46 ± 0·14 (7)	1·14 ± 0·12 (7)	1·75 ± 0·31 (7)	0·66 ± 0·12 (7)	1·53 ± 0·23 (7)	1·39 ± 0·09 (7)
1 (Expt. II)	0·33 ± 0·21 (7)	0·47 ± 0·22 (7)	0·06 ± 0·31 (7)	−0·04 ± 0·08 (6)	0·71 ± 0·15 (7)	0·20 ± 0·16 (7)
2 (Expt. I)	1·48 ± 0·13 (7)	2·79 ± 0·36 (7)	4·73 ± 0·45 (6)	0·81 ± 0·20 (7)	2·35 ± 0·30 (7)	4·37 ± 0·23 (7)
2 (Expt. II)	1·98 ± 0·20 (7)	4·10 ± 0·23 (7)	4·85 ± 0·48 (7)	1·09 ± 0·20 (7)	3·07 ± 0·20 (7)	4·92 ± 0·36 (7)
4 (Expt. I)	0·65 ± 0·17 (7)	3·70 ± 0·23 (7)	5·55 ± 0·25 (4)	0·12 ± 0·12 (7)	2·18 ± 0·19 (7)	4·36 ± 0·21 (7)
4 (Expt. II)	1·74 ± 0·18 (7)	3·30 ± 0·29 (7)	5·09 ± 0·27 (5)	0·93 ± 0·24 (7)	3·04 ± 0·17 (7)	3·92 ± 0·36 (7)
7 (Expt. I)	0·53 ± 0·18 (7)	1·64 ± 0·30 (7)	2·71 ± 0·14 (3)	−0·28 ± 0·16 (7)	1·50 ± 0·42 (6)	2·28 ± 0·25 (4)
7 (Expt. II)	0·24 ± 0·09 (7)	2·38 ± 0·19 (6)	3·61 ± 0·32 (3)	0·13 ± 0·17 (6)	1·66 ± 0·20 (5)	3·48 ± 0·33 (3)
	0·25 mg	1·0 mg	4·0 mg	0·25 mg	1·0 mg	4·0 mg
10* (Expt. I)	0·48 ± 0·19 (7)	0·99 ± 0·23 (7)	0·99 ± 0·20 (7)	0·12 ± 0·21 (7)	0·60 ± 0·17 (7)	0·72 ± 0·11 (7)
10* (Expt. II)	−0·31 ± 0·03 (6)	−0·19 ± 0·14 (7)	1·81 ± 0·39 (6)	0·10 ± 0·20 (6)	0·27 ± 0·12 (6)	0·67 ± 0·40 (7)

* In these experiments the dosage-range has been lowered in view of the high mortality as observed in the experiments after 7 days.

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TABLE 2. ACTIVITY RATIOS AT DIFFERENT TIMES AFTER ADMINISTERING

	Days after administering	Expt. I	Expt. II	Weighed mean value and P 0·95 interval
Dihydrotachysterol$_3$ / Dihydrotachysterol$_2$	1	(<2·5)	(±2·5)	—
	2	3·50	3·66	3·6 (76–131%)
	4	6·51	5·55	6·0 (75–134%)
	7	4·77	3·02	3·4 (74–135%)
Dihydrotachysterol$_2$ / Vitamin D$_3$	2	7·14	3·57	4·2 (76–131%)
	4	2·44	3·03	2·6 (76–132%)
	7	4·00	2·13	3·6 (73–136%)
Vitamin D$_3$ / Vitamin D$_2$	1	(>1·0)	(±1·0)	—
	2	1·48	1·67	1·6 (75–134%)
	4	1·93	1·90	1·9 (83–120%)
	7	1·76	1·32	1·4 (79–127%)
	10	(>1·0)	(>1·0)	—

From our results, it clearly follows that the ratios are highly dependent on the time interval. Concerning the vitamins D, we will point to the discrepancy which seems to exist between the two forms with respect to hypercalcemic and antirachitic activity. Vitamin D$_3$ showed in our experiments a higher hypercalcemic activity (of the order of ±1·6), while, with respect to their antirachitic activity, both vitamins D are equipotent in rats.

TABLE 3. ACTIVITY RATIOS (DIHYDROTACHYSTEROL$_2$ EQUAL TO ONE)

Days after administering	2	4	7
Vitamin D$_2$	0·15	0·20	0·20
Vitamin D$_3$	0·24	0·39	0·28
Dihydrotachysterol$_2$	1·0	1·0	1·0
Dihydrotachysterol$_3$	3·6	6·0	3·4

With rachitic rats, we determined the antirachitic activity of the two dihydrotachysterols, following a curative-test method.[3] For dihydrotachysterol$_2$, we found the antirachitic activity to be in the order of 0·5 per cent that of vitamin D, which value agrees with the value given by von Werder,[12] while dihydrotachysterol$_3$ shows an antirachitic activity of about 0·9 per cent, nearly twice as high as dihydrotachysterol$_2$.

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DIMETHANESULPHONOXYBUTANE (MYLERAN)
PRELIMINARY STUDIES ON DISTRIBUTION AND METABOLIC FATE IN THE RAT

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Abstract—(1) Dimethanesulphonoxybutane (Myleran) was synthesized with the sulphonoxy groups labelled with ^{35}S and, separately, with the butane moiety labelled with ^{14}C in the 1- and 4-positions.

(2) The distribution of isotopic drug following intravenous or oral administrations was determined in the rat. The specific activities of the organs at various times after administration of the drug were measured. The high specific activities of liver and kidney were thought to be related to the metabolic breakdown of the drug rather than to its assumed chemotherapeutic mode of action by alkylation of sensitive molecules.

(3) There was appreciable metabolic alteration of Myleran but some of the drug was excreted unchanged by the rat. The non-alkylating portion of the drug was excreted almost exclusively as methanesulphonic acid. The alkylating portion was partially degraded to carbon dioxide and to a compound with the characteristics of a glycol. Numerous metabolic products carried part of the isotopic carbon label. Part of this labelling appeared to be due to recycling of small carbon fragments and part to alkylation of normal constituents.

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INTRODUCTION

STUDIES on the metabolic fate and distribution of the cytotoxic alkylating agents following administration to mammals revealed that most of the compounds studied were rapidly removed from the blood following intravenous injection and were metabolized at a similar rate, so that in most cases no unchanged drug could be detected in the excreta.[1] Studies conducted with isotopically labelled methyl-^{14}C-bis(2-chloroethyl) amine HCl (HN2); triethylene-^{32}P-phosphoramide (TEPA); triethyleneimino-^{14}C-s-triazine (TEM); and bis-2-chloroethyl sulphide-^{35}S(sulphur mustard, H) indicated that the "alkylating" moiety of the drug molecule was detached from the carrier moiety; the latter then being excreted as cyanuric acid in the case of TEM or as inorganic phosphate in the case of TEPA.[2, 3] The radiocarbon of the alkylating portion of TEM appeared to be incorporated into numerous fractions which could then be isolated chromatographically from the urine. Only a few of these fractions were found to be normal constituents of the body.[2]

It was therefore of interest to investigate whether or not Myleran (dimethanesulphonoxybutane), an alkylating agent producing biological effects related to those of

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HN2 and TEM[4] but with somewhat different chemical properties[5] would follow a metabolic pattern similar to that observed with the other alkylating agents in current clinical use. This report deals with the synthesis, uptake, distribution and metabolic fate of Myleran-^{14}C and Myleran-^{35}S in the rat.

MATERIALS AND METHODS

Syntheses

The synthesis of dimethylsulphonyl-1:4-^{14}C-butanediol (Myleran-^{14}C) was as follows:

$$Na^{14}CN + Br{\cdot}CH_2{\cdot}CH_2{\cdot}Br \rightarrow N^{14}C{\cdot}CH_2{\cdot}CH_2{\cdot}{}^{14}CN$$

$$\downarrow$$

$$H_5C_2OO^{14}C{\cdot}CH_2CH_2{\cdot}{}^{14}COO{\cdot}C_2H_5 \leftarrow HOO^{14}C{\cdot}CH_2{\cdot}CH_2{\cdot}{}^{14}COOH$$

$$\downarrow$$

$$HO{\cdot}{}^{14}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}{}^{14}CH_2{\cdot}OH + CH_3SO_2Cl \rightarrow$$

$$CH_3{\cdot}SO_2{\cdot}O{\cdot}{}^{14}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}{}^{14}CH_2OSO_2CH_3$$

To 4 m-moles of sodium cyanide-^{14}C (0·25 mc/m-mole) in water, 3 m-moles of ethylene dibromide in absolute alcohol were added. The nitrile formed after heating was converted to sodium succinate by refluxing with NaOH at 105°C. Excess ethylene bromide was extracted with ether and acidified to pH 1 with HCl. After partial evaporation the succinic acid was isolated by continuous extraction with ether, and converted to the ethyl ester by heating with ethanol, toluene and conc. H_2SO_4 gradually from 90 to 125°C.

The crude diethylsuccinate dissolved in ether, was added to a suspension of 4 m-moles of lithium aluminium hydride in ether and refluxed. HCl was added and the 1 : 4-butanediol-1 : 4-^{14}C extracted with ether. After evaporation of the solvent, the diol was coupled with methanesulphonyl chloride according to the method of Timmis[6] in 1 ml of pyridine with equivalent amounts of methanesulphonyl chloride. Water was added at −10°C, the precipitate was filtered, washed twice with cold water, dried over phosphorus pentoxide and recrystallized from acetone–ether. The melting point was found to be identical with that of a sample of Myleran obtained from the Burroughs Wellcome Research Laboratories (115·5°C) and the specific activity of the drug assayed to $2{\cdot}1 \times 10^6$ counts/min per mg.

The synthesis of dimethylsulphonyl-^{35}S-1 : 4-butanediol (Myleran-^{35}S) is depicted as follows:

$$Ba^{35}S \rightarrow K_2{}^{35}S \rightarrow KCN^{35}S \rightarrow CH_3{}^{35}SCN \rightarrow CH_3{}^{35}SO_2Cl + HO{\cdot}CH_2{\cdot}CH_2CH_2{\cdot}CH_2OH \rightarrow$$

$$CH_3{}^{35}SO_2{\cdot}O{\cdot}CH_2CH_2CH_2{\cdot}O{\cdot}{}^{35}S{\cdot}O_2{\cdot}CH_3$$

Barium sulphide-^{35}S (5 m-moles) was converted to potassium thiocyanate, essentially by the Eldjarn[7] procedure, with a yield of approximately 80 per cent. Saturated KOH and subsequently dimethylsulphate were added to the stirred mixture. The mixture was stirred for 2 hr at room temperature, then for 15 min at 100°C, then cooled to −10°C and neutralized with concentrated HCl. The $CH_3{}^{35}SCN$ was oxidized and chlorinated in the cold by passing in chlorine. The methanesulphonylchloride was extracted with ether and the extracts washed with $NaHCO_3$. The bicarbonate washings were re-extracted with ether and the combined organic phases dried over sodium

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sulphate. The ether was distilled off and the methanesulphonylchloride esterified with a slight excess of 1 : 4-butanediol as described above. The initial specific activity of this compound was $2{\cdot}7 \times 10^6$ counts/min per mg.

Methods

Unless otherwise stated, adult male Osborn–Mendel rats were fasted 16 hr prior to administration of the drug. The drug was administered either orally or in a 10% acetone solution into the exposed femoral vein. Blood was obtained from the animal either at the end of the experiment by cardiac puncture, or if several blood samples were to be taken at frequent intervals after injection, through a polyethylene tubing inserted into the abdominal aorta several days previously. The various organs from the animals were excised immediately after sacrifice and either stored at −10°C or worked up immediately. Organ distribution of radioactivity at various times after the injection was estimated in two animals for each interval.

Aliquots of the organs were finely ground in a Potter–Elvejhem homogenizer and the homogenate made up to constant volume. Samples of 100 and 250 μl each were plated on weighed aluminium planchets. The planchets were counted after drying in a desiccator. Correction for self-absorption was applied on 1 mg tissue per cm^2 weight basis.

The organs were homogenized and the fat extracted with ethanol and then with an ethanol–ether mixture. The sodium nucleates were extracted with 10% sodium chloride solution at 85°C for 6 hr and again for 2 hr. Then 2·5 vols. of 95% ethanol were added to the pooled extracts at room temperature. After agitation with a glass rod, the precipitated sodium nucleates were separated by centrifugation. After redissolving in distilled water, the material was twice reprecipitated and finally dried with ethanol–ether and with ether and weighed. The extracted organs were dried with ethanol–ether and their dry weight determined.

In metabolic studies the animals were housed in a closed system which allowed for the collection of expiratory carbon dioxide in a series of scrubbing towers charged with sodium hydroxide solution. From the NaOH solution the carbonate was precipitated as the barium salt.[8] Aliquots of the dried and weighed barium carbonate were assayed for radioactivity.

Radioactivity was measured in a Radiation Counter Laboratories proportional gas flow counter. Corrections for self-absorption were made where necessary. In the case of sulphur-35, the amount of radioactivity measured was corrected for radioactive decay. Derivatives of radioactive metabolic products were prepared by the isotope dilution technique and recrystallization to constant specific activity. The following methods were employed in the identification procedures: 1 : 4 butanediol as the pseudosaccharin ether (m.p. 267–273°C) recrystallized from nitrobenzene;[9] amines by the Hinsberg procedure with *p*-toluenesulphonyl chloride;[10] oxalic acid as the calcium salt; sugars as the osazones;[10] urea as the xanthydrol derivative;[11] acetate as the acetylsulphanilamide (m.p. 218°C);[12] succinic acid as the succinyl sulphanilamide[13] (m.p. 213°C) (the reference compound was prepared according to Miller *et al.*;[14] sulphate on phosphate free urine as the benzidine derivative; methanesulphonic acid as the phenylhydrazonium salt (m.p. 193–194°C);[15] glycols by periodate oxidation.[10] Glycols were located on paper chromatograms by spraying with ammoniacal silver nitrate.

RESULTS

Myleran-^{14}C

Assuming the total blood volume to be 7 per cent of body weight, it was calculated that after a dose of 4·0 mg of Myleran-^{14}C per kg about 20 per cent of the administered drug radioactivity was retained in the circulation after 30 sec. After 60 min this amount had fallen to 2–4 per cent (see Fig. 1). The main decrease in blood radioactivity

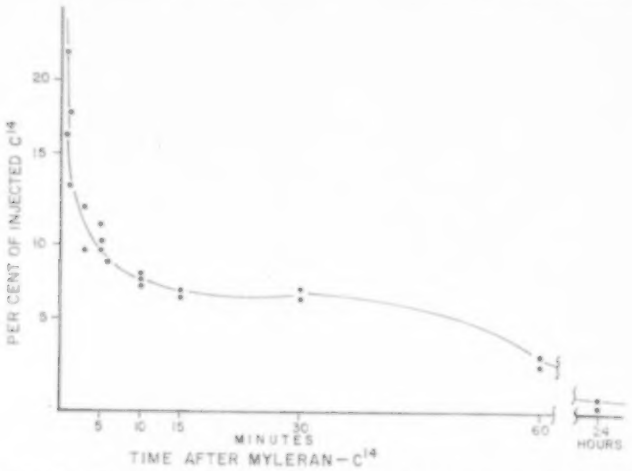

Fig. 1. Blood levels of radioactivity after intravenous injection of Myleran-^{14}C (calculated on the basis of specific activity of serum and expressed in per cent of injected dose recovered in whole blood).

occurred precipitously shortly after the injection of the drug. Less than 10 per cent of the blood radioactivity was associated with the formed elements. As shown in Table 1, the specific activity of livers, lungs and kidneys exceeded those of other organs.

Chemical fractionation of the liver was carried out in an attempt to localize major drug fragments by their isotopic label. Since a relatively high amount of $^{14}CO_2$ was

Table 1. Specific activity of various organs after intravenous administration of labelled Myleran to rats (4 mg/kg)

Organ	Myleran-^{14}C					Myleran-^{35}S
	Time after injection					
	1 min	10 min	15 min	60 min	24 hr	24 hr
Blood	0·490	0·327	0·303	0·182	0·038	
Liver	0·682	0·734	0·486	0·269	0·302	0·045
Kidney	0·627	1·497	0·553	0·868	0·568	0·059
Lungs	0·602	0·706	1·034	0·336	0·079	0·047
Stomach	0·469	—	0·237	—	—	
Small intestine	0·317	0·433	0·295	0·100	0·093	0·067
Colon	0·303	—	0·232	—	—	
Pancreas	0·552	—	0·156	—	—	
Brain	—	—	0·398	0·179	0·046	0·057
Muscle	0·175	0·247	0·256	0·147	0·038	0·056
Thymus	0·400	0·436	0·318	0·226	0·071	0·019
Spleen	0·543	0·217	0·269	0·394	0·145	0·074

Values are expressed in per cent of injected radioisotope recovered per gramme of wet tissue.

produced metabolically which would lead to appreciable recycling of one-carbon fragments over longer periods of time, only the livers of those animals were fractionated which had been sacrificed within 10 min after drug injection. Most of the radio-carbon was not yet incorporated by the tissue and could be readily extracted with ethanol and 10% TCA. The amount of radio-carbon isolated with the proteins, for instance, varied from 1 to 3 per cent of total organ activity. No radioactivity was recovered with the sodium nucleates. The amount of radioactivity recovered with the fats was not identified further, but some could have represented unchanged drug since also with the ^{35}S labelled Myleran small amounts of radioactivity were recovered in this fraction.

Radioactivity was measured in the expired carbon dioxide, the urine and the faeces after injection of the drug. Within 24 hr from 12 to 14 per cent of the injected ^{14}C was excreted as respiratory carbon dioxide-^{14}C. The specific activity of the exhaled $^{14}CO_2$ initially and at the end of 24 hr was low, showing a maximum about 6 hr after injection. Fig. 2 represents total $^{14}CO_2$ excretion and the specific activities thereof for a 24 hr period following drug administration.

The urinary excretion of isotopic carbon varied from 22 per cent to 36·4 per cent and the faecal excretion varied from 0·8 to 3·5 per cent for a 24 hr period following

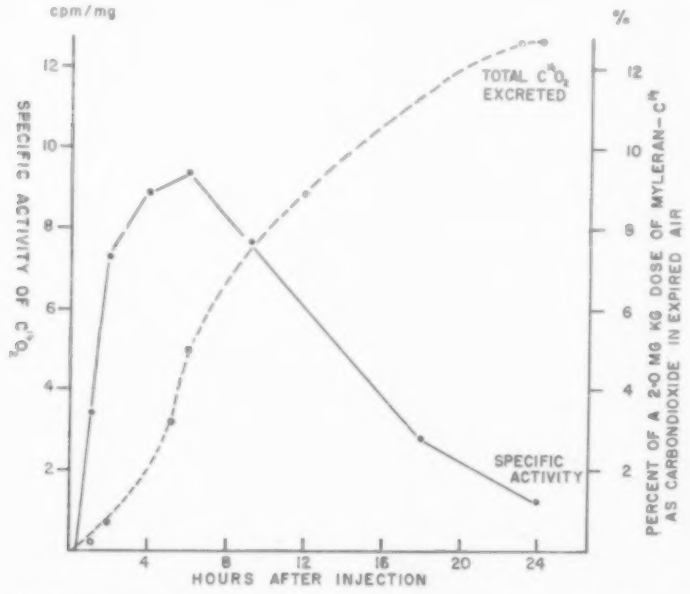

FIG. 2. Total excretion of carbon-^{14}C-dioxide and the specific activity thereof following intravenous Myleran-^{14}C.

intravenous injection of Myleran-^{14}C; after an equivalent oral dose it did not exceed 8 per cent in the urine for the same period. Thus, a total of up to 50 per cent of the injected radioactivity was recovered within a 24 hr period. An attempt was made to separate the radioactive urinary constituents by ion exchange chromatography. Dowex-50 columns 2 × 60 cm were eluted first with citrate buffers of increasing pH and then with sodium hydroxide of increasing strength. Complete recovery of urinary

radioactivity placed on the column was ascertained. As illustrated in Fig. 3, three active fractions were eluted with the initial washings, accounting for 70 to 80 per cent of the total radioactivity present. Thereafter, at least seventeen minor products were separated at various pH values. The three initial major peaks were rechromatographed on a Dowex-1 column and again were recovered with the first washings, this time at an alkaline pH. When percolated through a mixed bed column, between 70 and 80 per cent of the radioactivity was recovered from the eluate. These data indicated that the major urinary metabolites were neutral. By isotope dilution and paper chromatography one of the neutral peaks was identified as unchanged Myleran. One other major neutral peak, as isolated from the column, was found to be volatile. Solvent

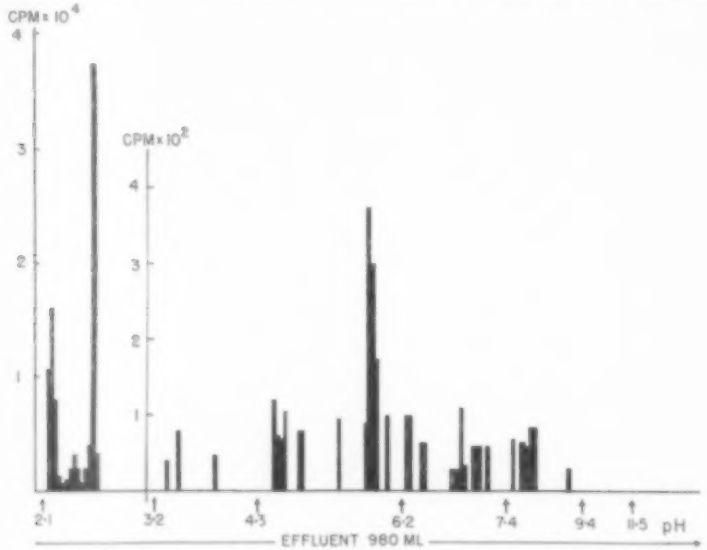

Fig. 3. Separation of radioactive urinary constituents on a buffered Dowex-50 ion exchange column after injection of Myleran-^{14}C.

partition studies indicated that this compound was quite polar, being only very slightly soluble in ether or chloroform or other non-polar solvents. Basic lead acetate, mercuric acetate, silver nitrate, 2 : 4-dinitrophenylhydrazine or phenylisothyanate failed to precipitate this product. Attempts to prepare a derivative with various diol carriers which could be recrystallized to constant specific activity were unsuccessful. Periodate oxidation and oxidation with bromine to CO_2 failed to yield enough labelled radioactive carbon dioxide to justify the assumption of adjacent hydroxyl groups in the metabolic product. Urine as well as the separated major metabolic product were subjected to permanganate oxidation or oxidation with H_2O_2 at various pH levels. The product obtained appeared to be less volatile. The oxidized product was not identical to any one of several mono- or di-carboxylic acids co-chromatographed (Table 2).

Paper chromatography indicated the presence of 1 : 4-butanediol as a minor metabolic product of Myleran. By recrystallization with 1 : 4-butanediol-carrier some radioactivity could be isolated from the second major peak as the pseudosaccharin ether. This, however, did not amount to more than 5 per cent of the administered

dose. Urea isolated as the xanthydrol derivative accounted for 5 per cent of the urinary activity. In addition, the presence of small amounts of radioactive glucose, oxalate and amines could be demonstrated.

A more detailed study was made of the relative amounts of Myleran excreted unchanged in rats following a dose of 2 mg/kg. Approximately 60 per cent of the excreted

TABLE 2. R_f VALUES OF VARIOUS GLYCOLS, RELATED ALIPHATIC ACIDS AND R_f VALUES OF THE MAJOR URINARY METABOLITE AND OXIDATION PRODUCTS THEREOF

Compound	Solvent system no. 1	2	3	4
Myleran-^{14}C	0·82	0·95	0·95	—
Neutral, volatile urinary product	0·56	0·80	0·38	—
1 : 4-Butanediol	0·67	streak	0·38	—
1 : 3-Butanediol	0·73	—	0·39	—
2 : 3-Butanediol	0·77	—	0·47	—
1 : 2-Propanediol	0·69	—	0·15	—
1 : 3-Propanediol	0·71	—	0·28	—
1 : 2-ethanediol	0·66	—	0·07	—
Oxidized urinary met. product	0·60	0·80	—	0·78
Oxalic acid	0·55	0·23	—	streak
Succinic acid	0·73	0·80	—	0·70
Malonic acid	0·73	0·70	—	0·67
Glycolic acid	0·68	0·57	—	0·59
Lactic acid	—	0·70	—	0·72
Malic acid	0·66	0·52	—	0·51
Tartaric acid	0·55	0·38	—	0·39

Solvent systems: no. 1—95 per cent methanol; no. 2—methylethylketone 75 : propionic acid 25 : water 30; no. 3—ethyl ether–chloroform 1 : 1 sat. with H_2O; no. 4—butanol–ethanol 1 : 1.

radioactivity during the first 6 hr could be accounted for as unchanged drug; 25 per cent during the 6–12 hr period, which decreased then to only 5 per cent during the 12–24 hr period.

The relatively high proportion of carbon-14 in the expiratory carbon dioxide and its occurrence in common metabolites such as urea and glucose suggested the possible degradation of the 4-carbon chain into smaller fragments. Since hydrolytic cleavage of Myleran would yield the 1 : 4-butanediol, this compound was administered orally to rats in combination with an oral dose of Myleran-^{14}C. No changes in the urinary excretion of ^{14}C or in the specific activity of expired carbon dioxide could be observed, even with LD_{50} doses (0·5 g/rat) of butanediol. This suggests that intermediary formation of the 1 : 4-diol was unlikely. This avenue was explored further, however, by investigating the succinic acid pathway derived from the butane moiety of Myleran. Myleran-^{14}C was injected intravenously into three rats, one of which was pretreated with a dose of 100 mg of oral sulphanilamide in oil and 100 mg malonate; another received sulphanilamide only and a third received Myleran only. The urines from all three animals were collected and the excretion of ^{14}C was measured throughout 144 hr. The rats which had received sulphanilamide excreted twice the amount of radioactivity during the first 6 hr as the controls. However, the overall 144 hr excretion

of ^{14}C was quite similar in all three rats. From the first animal the urinary succinylsulphanilamide was isolated and acetylsulphanilamide was isolated from the second rat. After decolorization with charcoal, the conjugates were recrystallized from six to eight times, the melting points were determined and the specific activity of each was calculated. The amount of radio-carbon recovered in the acetyl- or succinylsulphanilamides was less than 0·06 per cent of the administered dose.

Myleran-^{35}S

Blood concentrations of radioactivity after intravenous injections of Myleran-^{35}S closely resembled those obtained with the carbon-14 labelled drug. As shown in Table 1, 24 hr after the administration of the drug there appeared to be selective concentration of radio-sulphur in the intestines and spleen. However, the specific activity of the organs was too low to permit determination of the nature of the ^{35}S-containing constituents. Nearly 30 per cent of the activity localized in these organs could be removed by extraction with alcohol–ether. Following a dose of Myleran-^{35}S of 2·0 mg/kg, 86 per cent of the radio-sulphur was excreted in the urine after intravenous injection, 60 per cent after intraperitoneal injection and 62 per cent after oral administration in 24 hr. Partitioning of the urinary radioactivity in three different solvent systems yielded two different peaks of radioactivity, one of which coincided with the R_f value of methanesulphonic acid (Table 3). The identification of methane-

Table 3. R_f values of Myleran-^{35}S, urinary ^{35}S metabolite and methanesulphonic acid in different solvent systems

Compound	Solvent system no. 1	5	6
Myleran-^{35}S	0·82	0·84	0·96
Rat urine-^{35}S	0·80	0·10	0·17
Methanesulphonic acid	0·80	0·11	0·17

Solvent systems: no. 1—95 per cent methanol; no. 5—*n*-butanol–AcH–H_2O, 4 : 1 : 1; no. 6—acetone–AcH–H_2O, 8 : 1 : 1.

sulphonic acid was carried out as follows: An aliquot of the urine was continuously extracted with ether for 30 hr. The ether extract was evaporated to dryness and dissolved in from 2 to 4 ml of water. Subsequently 100 to 200 mg of methanesulphonic acid carrier were added and the phenylhydrazonium derivative of methanesulphonic acid was prepared. After repeated recrystallizations the specific activity of the derivative remained constant. The melting point of the derivative prepared from known methanesulphonic acid was 196·5 °C and a mixed melting point showed no depression. It was feasible to prepare the phenylhydrazonium derivative of methanesulphonic acid directly from urine by adding known amounts of carrier compound to urine and recrystallizing the derivative.

In several experiments it was shown that from 62 to 68 per cent of the urinary radioactivity was present as methanesulphonic acid. Carrier recrystallizations with added Myleran indicated that another 25 to 30 per cent of radio-sulphur in the urine could be accounted for by unchanged drug. Only from 0·03 to 0·04 per cent of the

urinary ^{35}S, measured as the benzidine derivative, was present in the form of inorganic sulphate. From 5 to 8 per cent of the urinary ^{35}S remained unidentified or not accounted for.

DISCUSSION

The rapid disappearance of the Myleran from the circulation after injection followed the pattern observed with other alkylating agents.[16] With the ^{14}C-labelled drug there appeared to be higher concentration of isotope within liver, kidneys and lungs; however, a "selective uptake" by these tissues was unlikely. It is postulated that the higher specific activities of these organs were due to ^{14}C-containing fragments which arose during the metabolic breakdown of the drug. This hypothesis is supported by the finding that appreciable amounts of the 1 : 4-^{14}C-butane chain were recovered as carbon-^{14}C-dioxide. Similarly, the small to moderate amounts of label recovered in urinary glucose, oxalate and urea may have arisen in the liver or kidneys, perhaps derived secondarily from one- or two-carbon fragments obtained from the drug.

Comparison of the specific activities of organs at 24 hr following the injection of either the sulphur- or the carbon-labelled Myleran showed that the spleen had a high amount of isotope incorporated per gramme of wet tissue. Peng, who reported the uptake of ^{35}S from labelled Myleran by the different organs, found that spleen concentrated the isotope from two- to sevenfold over other organs. He inferred that this was "probably a result of the selective affinity this organ possesses for the chemical".[17] The findings reported here neither support nor refute this contention since the identity of the isotopic material in the spleen could not be established. Because the specific activity of the spleen was only about one-half to one-fourth that of liver or kidney in the case of carbon-14 and exceeded that of liver and kidney only by 67 and 26 per cent respectively in the case of sulphur-35, reasonable doubt remains as to the therapeutic implications of this incorporation.

The overall metabolic pathways of Myleran were only partially established. From the Myleran-^{35}S injected rats the urinary radio-sulphur was recovered almost quantitatively in the forms of methanesulphonate and unchanged drug. The small yield of inorganic ^{35}S indicated that only minute amounts of methanesulphonate were split further. The relatively high yield of $^{14}CO_2$ from Myleran-^{14}C established that a breakdown of the butane chain occurred following the initial metabolic cleavage of the drug. It is assumed that the numerous small radioactive fractions separated chromatographically, among others, represented normally occurring metabolic products. The occurrence of alkylated amino acids, etc., similar to those observed by Roberts *et al.*[18] with the half-Myleran, was considered possible, but could not be demonstrated because of the low activity of these fractions.

Since concomitant injection of sulphanilamide together with Myleran-^{14}C led to the recovery of only negligible amounts of conjugated acetate-^{14}C or succinate-^{14}C, degradation of the butane moiety was assumed to take place via pathways other than through cleavage into a two-carbon fragment or oxidation into succinate and subsequent entrance into the tricarboxylic acid cycle. Also the simultaneous adminstration of 1 : 4-butanediol with Myleran failed to have any influence on the metabolic pattern of the drug, indicating that metabolism via 1 : 4-butanediol was not a major pathway. The major metabolic product of Myleran-^{14}C was found to be neutral and volatile and exhibited the characteristics of a glycol. The possibility that this metabolite

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was an oxidized monoester was considered, but an identification could not be established.

From the foregoing observations it was concluded that Myleran essentially followed a metabolic pattern similar to that established for the other alkylating agents studied. The methanesulphonyl moiety of the drug is readily removed from the butane chain as indicated by the high yield of methanesulphonate on the urine of the rat. Both with sulphur- and with carbon-labelled Myleran excretion of some unchanged drug in the urine was observed in rats. Although these alkylating agents were reported to be quite reactive under biological conditions, relatively high dosage schedules can result in the excretion of unchanged drug as reported also by Maller and Heidelberger.[19]

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SOME BIOCHEMICAL EFFECTS OF 2-ETHYLAMINO-1, 3, 4-THIADIAZOLE

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Abstract—It has previously been demonstrated (Kaplan *et al.*[4]), that the injection of large doses of nicotinamide results in a ten-fold increase in the diphosphopyridine nucleotide (DPN) content of the liver of mice. The current report shows that the nicotinamide antagonist 2-ethylamino-1,3,4-thiadiazole (EAT) does not behave as a competitive inhibitor of nicotinamide in the induced synthesis of DPN *in vivo*. A high dose of EAT (500 mg/kg) inhibited DPN-synthesis from a high dose of nicotinamide (500 mg/kg) by 30 per cent. However, with a low dose of nicotinamide (125 mg/kg), stimulation of DPN-synthesis was as high as 70 per cent. With or without low doses of nicotinamide, EAT increased the incorporation of formate-^{14}C and of glycine-2-^{14}C into acid-soluble liver-adenine. Evidence was obtained that EAT may interfere with the urinary excretion of nicotinamide. The relation of this effect to the above observations is discussed.

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THE chemotherapeutic activity of a number of 1,3,4-thiadiazoles against several mouse neoplasms was reported by Oleson *et al.*[1] In subsequent work it was demonstrated that these compounds act as antimetabolites of nicotinamide in bacteria and in mice.[2] Both the anti-tumor activity and the toxicity of 2-amino–1,3,4-thiadiazole can be prevented by treatment with nicotinamide.[2, 3]

Because of this relationship to nicotinamide it was considered that the thiadiazoles might act by interfering with the synthesis or function of the nicotinamide-containing coenzyme, diphosphopyridine nucleotide (DPN). Ciotti *et al.*[3] have reported that the enzyme diphosphopyridine nucleotidase can catalyze the synthesis of thiadiazole analogues of DPN by an exchange reaction. The present report deals with the effect of 2-ethylamino-1,3,4-thiadiazole (EAT) on the synthesis *in vivo* of DPN from nicotinamide.

When mice are injected with large doses of nicotinamide there is a temporary ten-fold increase in the DPN-content of the liver.[4] A number of nicotinamide antagonists are known to inhibit this synthesis of DPN *in vivo*.[5] The interpretation of experiments with substituted pyridine antagonists such as 3-acetyl pyridine is difficult because many of these compounds are themselves precursors of DPN in the whole animal.[5] This difficulty would not be anticipated in the case of thiadiazole derivatives.

While EAT inhibited the synthesis of DPN from high doses of nicotinamide, it actually stimulated DPN-synthesis from low doses of nicotinamide. This paper describes the quantitative aspects of these relationships.

In previous work it was observed that doses of nicotinamide which cause an increase

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in liver DPN also stimulate the incorporation of formate and glycine into acid-soluble liver adenine, presumably by increasing the synthesis *de novo* of adenine required for making DPN.[6] Although EAT is a nicotinamide antagonist, at low doses of nicotinamide it actually stimulated incorporation of formate and glycine into acid-soluble liver adenine. In control experiments, in which no nicotinamide was given, it was observed that EAT, by itself, also stimulated formate-incorporation into acid-soluble adenine. Some of the features of this stimulation are described in the present paper.

MATERIALS AND METHODS

The mice used in these experiments were 3-month-old male (BALB/c An × DBA/2J) F_1 hybrids which weighed 22 to 27 g and were maintained on a Purina chow diet. The thiadiazole derivative (EAT-HCl) was obtained from the Cancer Chemotherapy National Service Center of the National Cancer Institute and through the courtesy of the American Cyanamid Company. It was injected in aqueous solution, adjusted to pH 7 with sodium bicarbonate. From the time of injection to sacrifice the mice were allowed water, but no food.

The mice were killed by cervical fracture. Livers were homogenized with 5 volumes of 5% trichloroacetic acid (TCA) and the DPN-content of the TCA-extracts was assayed with alcohol dehydrogenase according to Ciotti and Kaplan.[7] EAT had no effect on this assay at a concentration of 1·7 mg/ml.

For collection of urine, mice were kept in beakers on wire grids over filter paper. The filter papers were removed at different times and eluted with 50 ml of water. An aliquot of the eluates was plated on glass planchets for determination of radioactivity. Sodium formate-^{14}C and glycine-2-^{14}C were purchased from commercial sources and had activities of 1–2 μc/μmole. Carboxyl-labeled nicotinamide-^{14}C was obtained from New England Nuclear Corporation. Radioactivity compounds were administered at a level of 5 μc/mouse, unless otherwise indicated.

Total acid-soluble adenine was isolated as the silver salt following hydrolysis of a 5% TCA-extract in 1 N H_2SO_4 for 1 hr at 100°.[8] Silver was removed with hot 0·3 N HCl and the free adenine was purified by chromatography in methanol: conc. HCl: water (100:34:66).[9] After elution from paper chromatograms with 0·1 N HCl the adenine concentration was determined according to Bendich,[10] and an aliquot was plated for measurement of radioactivity in a gas-flow proportional counter.

The precipitate obtained after the liver was homogenized in 5% TCA was washed twice with cold 5% TCA, then extracted with hot 5% TCA at 100° for 30 min to remove nucleic acids.[11] The residue was washed with water, then with ethanol, and dried with ether. A sample was dissolved in hot formamide and an aliquot containing 1·0 mg of protein was dried on a stainless steel planchet under an infrared lamp for measurement of radioactivity.

RESULTS

Effect of EAT on DPN synthesis from different doses of nicotinamide

In preliminary work it was felt that EAT might act as a competitive antagonist of nicotinamide in the induced synthesis of DPN. If this were the case, the extent of inhibition would depend on the ratio of EAT to nicotinamide. However, DPN-synthesis from low doses of nicotinamide was not inhibited by EAT while inhibition

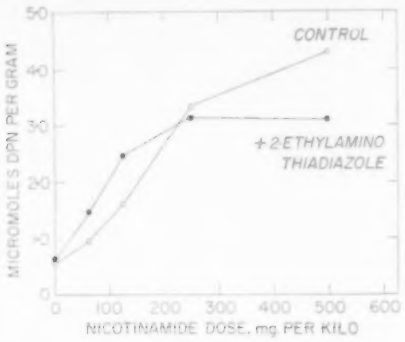

FIG. 1. Effect of 500 mg of EAT per kg, on the amount of synthesis of DPN resulting from different doses of nicotinamide. EAT was administered subcutaneously 10 min before the injection of nicotinamide (0·01 ml/g body weight) intraperitoneally. Mice were killed 6 hr after nicotinamide injection. Each point represents the average obtained from the results of analyses of samples from three mice.

of synthesis from high doses was quite evident. As shown in Fig. 1, 500 mg of EAT per kg of body-weight actually seemed to stimulate DPN-synthesis from doses of nicotinamide less than 250 mg/kg. EAT did not alter the DPN-content of the liver when no nicotinamide was given. These results suggest that under the conditions used in this experiment EAT did not behave as a competitive antagonist of nicotinamide for DPN-synthesis.

To determine whether the apparent stimulation by EAT of DPN-synthesis from 125 mg of nicotinamide per kg was significant, the effect of different doses of EAT was measured. As shown in Fig. 2, maximal stimulation was obtained with 250 mg of EAT per kg.

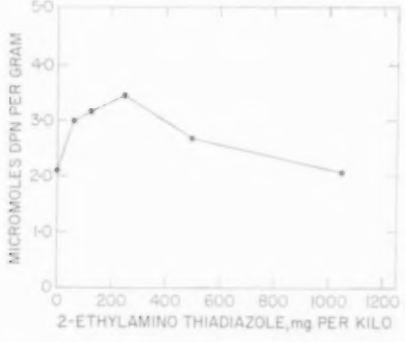

FIG. 2. Effect of different doses of EAT on DPN synthesis from 125 mg of nicotinamide per kg. Conditions were the same as for Fig. 1.

Time relationships

The stimulation of DPN-synthesis by nicotinamide is temporary; within 24 hr after the injection of nicotinamide, the levels of DPN return to normal.[4] When low doses of nicotinamide are given, peak levels of DPN may occur several hr earlier than when a high dose is given. Figure 3 shows the effect of two doses of EAT on the synthesis of

DPN from 125 mg of nicotinamide per kg. There was definite stimulation of DPN-synthesis by 250 and by 500 mg of EAT per kg, so that the amount of DPN synthesized began to approach that obtained by injecting 300 to 500 mg of nicotinamide per

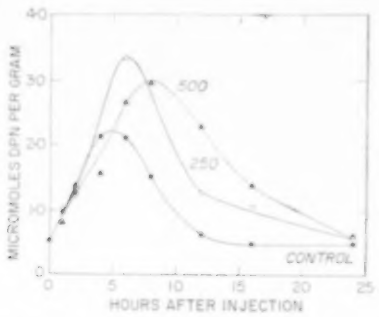

FIG. 3. Effect of EAT on DPN synthesis during 24-hr period. Conditions were the same as for Fig. 1. Groups of 3 mice were killed at various times after injection of nicotinamide (125 mg/kg). The dosage of EAT was 250 and 500 mg/kg.

kg. There was also a shift in the time when the peak concentration of DPN occurred, so that the curves obtained resembled those observed when higher doses of nicotinamide were given without EAT.

Because of the possibility that the injection of EAT might produce some change which could affect the alcohol dehydrogenase assay system the isolation of DPN was carried out in one experiment. Acetone precipitation and chromatography on Dowex-1-chloride[12] was used to obtain DPN from a 5 per cent TCA extract of the livers of 10 mice killed 6 hr after the injection of nicotinamide (125 mg/kg) and 500 mg/kg of EAT. The control group was injected with nicotinamide but no EAT.

The material thus isolated was concentrated under reduced pressure at room temperature and its DPN-content was assayed with alcohol dehydrogenase and by the cyanide method.[7] The yield of DPN as determined by both these methods was 12·7 μmoles for the group of mice receiving nicotinamide alone, and 21·2 μmoles for the group injected with both nicotinamide and EAT. In each case this represents about 60 per cent of the DPN extracted from the livers with 5 per cent TCA as determined directly with alcohol dehydrogenase.[7] This is the yield usually encountered in the precipitation of small amounts of DPN with acetone. Upon chromatography in the ammonium acetate–ethanol system of Paladini and Leloir[13] the liver-DPN gave a single ultraviolet quenching spot with the same mobility as authentic DPN.

Effect of EAT on nicotinamide excretion

Mice were injected with EAT and nicotinamide-$^{14}CONH_2$ ($2{\cdot}2 \times 10^6$ counts/min per mouse), under conditions which produced maximal stimulation of DPN-synthesis from 125 mg of nicotinamide per kg. In two experiments EAT (both 250 and 500 mg/kg) caused a decrease of about 50 per cent in the urinary excretion of radioactivity during the first 6 hr. Although recovery of radioactivity in the urine was not extensive (approximately 15 per cent in controls), the results suggest that the nicotinamide antagonist may contribute to the increased effectiveness *in vivo* of a low dose of nicotinamide by interfering with the excretion of the latter.

Effect of EAT and nicotinamide on formate and glycine incorporation

The incorporation of formate-^{14}C and glycine-2-^{14}C *in vivo* into the acid-soluble adenine of mouse liver was found to be increased by the injection of doses of nicotinamide which produce large increases in the DPN-content of the liver.[6] Smaller doses (below 300 mg/kg) have much less effect. As in the case of DPN-synthesis, a low dose of nicotinamide when given together with 500 mg of EAT per kg stimulated the incorporation of formate and of glycine as much as a high dose of nicotinamide alone (Table 1). With 500 mg of nicotinamide per kg, EAT did not stimulate the incorporation of formate, but actually inhibited it. While these effects might be correlated with changes in DPN-synthesis, the increased incorporation of formate observed when mice were given thiadiazole alone was quite unexpected, and was examined in greater detail.

TABLE 1. EFFECT OF EAT PLUS NICOTINAMIDE ON FORMATE AND GLYCINE INCORPORATION

Nicotinamide (mg/kg)	EAT (mg/kg)	Liver DPN (μmoles/g)	Acid-soluble, liver adenine (counts/min per μmole)	
			Formate	Glycine
0	—	0·51	600	785
125	—	1·81	910	1340
500	—	4·50	3100	2390
0	500	0·62	3067	1090
125	500	2·70	9050	4340
500	500	3·41	1180	4320

EAT was administered 5 min before nicotinamide. After 6 hr each mouse was injected with 5 μc of formate-^{14}C or glycine-2-^{14}C. Mice were killed 1 hr after injection of labeled compound. Each point represents the average obtained from the analyses of samples from two mice.

Effect of EAT on formate incorporation

Mice were injected with EAT (500 mg/kg) and then with formate-^{14}C. The time-course of the incorporation of the formate into acid-soluble liver adenine is shown in Fig. 4. While considerable variability was encountered, this stimulation of formate-

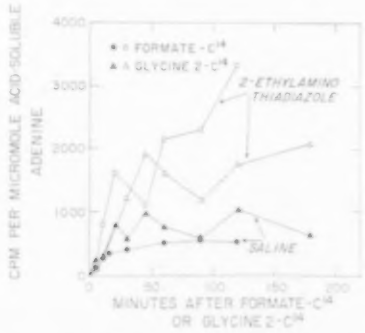

FIG. 4. Effect of EAT on the incorporation of formate and glycine into acid-soluble liver-adenine. EAT (500 mg/kg) was injected 5 min before formate-^{14}C or glycine-2-^{14}C. Controls were injected with saline.

incorporation was verified in four different experiments. The response to different doses of EAT is shown in Fig. 5. Maximal stimulation was observed with a dose of 500 mg/kg.

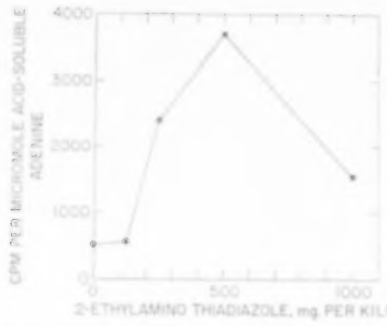

FIG. 5. Effect of different doses of EAT on formate-incorporation into acid-soluble liver-adenine. The mice were killed 1 hr after the injection of formate-^{14}C. EAT was administered 5 min before the formate-^{14}C.

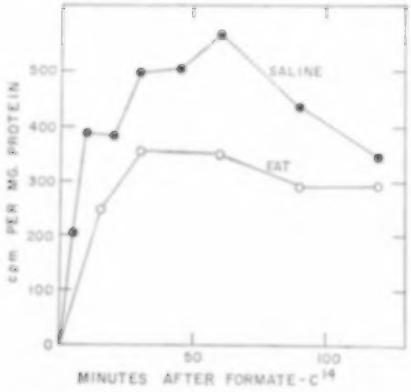

FIG. 6. Effect of EAT on formate incorporation into liver protein. Conditions were the same as for Fig. 4.

The stimulation of the incorporation of formate by EAT was not a general effect, since there was no increase in the incorporation of formate into liver proteins (Fig. 6).

Effect of EAT on glycine incorporation

As shown in Fig. 4, EAT produced some increase in the incorporation of glycine-2-^{14}C into acid-soluble liver adenine. Maximum stimulation was 2-fold, as compared with the 5- or 6-fold stimulation usually obtained for formate-incorporation. The uptake of glycine into liver protein was not appreciably affected by EAT.

DISCUSSION

Under the conditions used in these experiments it would appear that 500 mg of EAT per kg can inhibit by 30 per cent the synthesis of DPN *in vivo* from 500 mg of nicotinamide per kg. With lower doses of nicotinamide there was as much as 70 per cent

stimulation. These results suggest that EAT is not an active competitive antagonist of nicotinamide during the synthesis of DPN *in vivo*.

Since giving EAT together with a small dose of nicotinamide seems to allow the same amount of DPN-synthesis as a larger dose of nicotinamide alone, the possibility remains that EAT is interfering with the elimination of nicotinamide from the body. This could produce an apparent "sparing" action by allowing a more efficient utilization of nicotinamide *in vivo*. Experiments with carboxyl-labeled nicotinamide-^{14}C suggest that EAT may decrease the urinary excretion of low doses of nicotinamide. It would be of interest to determine whether this change would also be reflected in the maintenance of increased blood levels of nicotinamide following the injection of nicotinamide and EAT.

It has also been possible to obtain some biological evidence showing that EAT increases the effectiveness of low doses of nicotinamide. We have found in preliminary experiments that EAT will enhance the protection afforded by pre-treatment with low doses of nicotinamide against the toxicity of the nicotinamide antagonist 3-acetyl pyridine.

A combination of 500 mg of EAT and 125 mg of nicotinamide per kg increased the incorporation of formate and glycine into acid-soluble liver adenine in the same manner as 500 mg of nicotinamide per kg alone. The relationship of this increased formate-incorporation to DPN-synthesis is not clear, since the stimulation of the incorporation of formate by EAT alone cannot be attributed to any changes in the content of DPN.

A number of toxic compounds, such as carbon tetrachloride and N-methyl formamide, can increase the incorporation of formate (and other precursors) into the liver *in vivo*.[14] The increase observed in such cases has been correlated with liver damage, and occurs in the protein and glycogen, as well as in the adenine. The doses of EAT used in these experiments were not toxic and there was no gross evidence of liver damage such as can be obtained with carbon tetrachloride.

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SUMMARY

The nicotinamide antagonist 2-ethylamino-1,3,4-thiadiazole (EAT) does not appear to act as a competitive inhibitor of nicotinamide for the induced synthesis of mouse liver diphosphopyridine nucleotide (DPN) *in vivo*. EAT (500 mg/kg) caused a 30 per cent inhibition of DPN-synthesis from a high dose of nicotinamide (500 mg/kg), but increased by as much as 70 per cent the synthesis of DPN from a low dose of nicotinamide (125 mg/kg). EAT also increased the effectiveness of low doses of nicotinamide in stimulating the incorporation of formate-^{14}C and glycine-2-^{14}C into acid-soluble liver adenine. In part, these results may be attributed to the interference of EAT with the urinary excretion of nicotinamide. In addition, EAT by itself was found to stimulate the incorporation of formate-^{14}C, and, to a lesser extent, of glycine-2-^{14}C, into acid-soluble liver-adenine.

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FURTHER OBSERVATIONS UPON THE TOXIC PRINCIPLE OF *DICHAPETALUM TOXICARIUM*

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Abstract—The mixed fatty acids obtained from the seeds of *Dichapetalum toxicarium* by hydrolysis have been fractionated by low-temperature separation from organic solvents followed by reversed-phase chromatography. After further purification by a defined calcium precipitation, an oil of m.p. 13·0°C ± 1·0 was separated. The main constituent was a long-chain fluoroacid containing eighteen C atoms, one double bond and one F atom, i.e. a fluoro-octadecenoic acid. In biochemical tests with kidney particles, as little as 1·25 μg of the fluoro fatty acid led to citric acid accumulations. Some of the tests upon rats are recorded; it was found that at death there was usually more citric acid in the heart than in the kidney, which is the reverse of the picture with fluoroacetate. In terms of fluorine content, the long-chain fluoro-fatty acids are more toxic than fluoroacetate.

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IT has been reported by Peters *et al.*,[10] that the toxic principle in the seeds of *Dichapetalum toxicarium* behaves as a fluorine-containing fatty acid running like a C_{13} saturated fatty acid in reversed-phase chromatography. The acid was metabolized even in small amounts by kidney particles to a substance behaving like fluorocitric acid. To isolate and characterize this fluoro-fatty acid, the studies have been continued and extended with fresh batches of seeds from Sierra Leone.* The methods of reaching the active fraction for chromatographic analysis have now been shortened by fractionation at low temperatures (− 20°C) in light petroleum or acetone; the bulk of the fluorine-containing acid was in the most soluble fractions. By a further purification by column chromatography and then as a calcium salt, a fluorine-containing fatty acid has been isolated in small amounts containing eighteen C atoms and one double bond.

Some introductory remarks are necessary regarding the procedure. In the isolation of a toxic agent by fractionation, an exact LD_{50} is usually unnecessary. As the material was limited in this case, it was fortunate that the toxicity could be followed with very few, even single rats, by taking advantage of increasing experience of the clinical state of the animal. For instance, the bradycardia, which was a characteristic sign of intoxication, proved useful, as did the citrate determination after death.

METHODS

Biological and biochemical tests for toxicity

Toxicity tests with rats. Oral doses were used at the start, the ground seeds being made up into cubes with an equal or convenient amount of rat cake. Later, tests were made on the oily fractions by intraperitoneal injection (0·1 to 0·3 ml) in arachis oil, ethanol or acetone. The behaviour of the rats was closely observed; an early onset of

* Obtained through the kindness of the Colonial Products Research Council, Director, Dr. R. A. E. Galley.

nervous signs or of bradycardia indicated marked toxicity and further information was obtained from citrate analysis, expecially on heart and kidney; the accumulation of citric acid in the heart is more than that in the kidney with the longer chain fluoro-fatty acids Peters.[8, 13] Bradycardia was followed either by the stethoscope or by recording the heart sounds with a special microphone supplied by the Cambridge Instrument Company. For this type of observation, the auditory method is more convenient and causes less disturbance than taking electrocardiograms.

Biochemical tests. These were made with guinea pig kidney and pigeon brain particles as described elsewhere.[10] Small amounts of alcohol had to be used for solution.

Estimations and chemicals. Estimation of citric acid was made by the method of Taylor.[12] The chemicals used were "Analar" where possible. Glass-distilled water was used throughout.

Double bond estimations. The following method was adapted from that of Trappe:[11] 0·5 mg or less of fatty acid was dissolved in $CHCl_3$ (1 ml) and treated with 0·01 N bromine dissolved in methanol saturated with NaBr (2 ml). After standing 4–5 hr at room temperature, KI (1 ml of 2% w/v) was added; the iodine liberated was titrated with 0·002 N Na thiosulphate using as indicator 1% starch in 0·1 N borax solution (0·2 ml). Titrations were made with a microburette. A blank without the fatty acid was taken through the procedure. The difference in the thiosulphate determination gave the double bond value for the sample. Estimations were usually made in triplicate, and gave individual values varying within less than 1 per cent, if scrupulous attention was given to the measurement of fatty acid and bromine solutions.

Carboxyl titration. The fatty acid in 70% (v/v) acetone–water (2 ml) with 0·04% phenol red (0·02 ml) was titrated in a stream of N_2 with 0·01 N NaOH in 50% (v/v) acetone–water until the appearance of a red colour persisting for more than 30 sec. The accuracy was $\pm$ 5 μl in 300 μl.

Fluorine. This has been estimated in two ways: (1) by a spectrochemical method and (2) by titration after combustion.

(1) For the spectrochemical estimation, we are much indebted to Mr. F. T. Birks of AERE, Harwell, whose determinations have the advantage that they are made in the specific bands for F of 7128·0 Å and 7202·4 Å.

(2) A sample (weight approximately 1–3 mg) was fused in a Parr-type nickel bomb (gold washer) with sodium (50 mg) at 500°C for 1 hr; the solution was then titrated with a thorium nitrate solution. The method was essentially that of Armstrong[1] using a monochloroacetic acid buffer.[4, 6] Solochrome Brilliant Blue BS was the indicator. Taking into account the difficulties of estimating colour changes with Solochrome Blue even under the most favourable illumination, and using optimal amounts of F for microtitration (i.e. 10–15 μg), it was not found possible to estimate nearer than to 0·005 to 0·01 ml, i.e. $\pm$ 2 per cent in the titration. In addition, a blank given by Na alone in the bomb was found to vary from 0 to 0·02 ml. The recovery was 96 per cent for an amount of F between 50 and 100 μg. In our hands, therefore, these F estimations did not reach the detailed accuracy of the —CH = CH— determinations; in general this is true for F estimation. It may be mentioned that with pure Na fluoroacetate (2·673 mg) containing 508 μg F, recovery was quantitative after fusion in the bomb. With 10-fluorodecanoic acid (1·735 mg) containing 173·5 μg F, recovery was 96·5 per cent and 94 per cent on two occasions.

Reversed-phase partition chromatography. This was conducted in principle according to the technique of Howard and Martin,[7] the tubes used being 10 mm in internal diameter and 45 cm in length; five tubes were run in parallel at room temperature (cf. Crombie *et al.*[3]), but with no indicators. The use of larger tubes proved unsatisfactory for separating the fractions. At the top of the tubes there were separating funnels fitted into the tubes with polythene tubing so that an air-tight joint was made. A porous glass plate was fused about 3 in. (7·62 cm) from the bottom of the tubes, the ends of which were fitted with glass tubes 6 mm diameter and about 11·5 cm long drawn to a point which were placed closely inside the main chromatography tube with a sleeve of polythene tubing. Elutions were started with 55 or 60% (v/v) aqueous acetone. Each 2 ml was titrated with 0·01 N NaOH (in methanol) as it appeared, the indicator being phenol red. N_2 was passed through the acetone and titration fluid.

EXPERIMENTAL RESULTS

The method of isolation of the active principle was checked by animal tests and was continuously improved as the investigation progressed. Hence, one of the later fractionations will be described. This followed in the main standard methods of fractionation with solvents at low temperatures,[5] but it is to be regarded only as indicative of the method of getting the fatty acid fraction soluble at low temperatures, and it could be varied in detail. Of approximately sixty rats used, only ten tests are recorded in Table 1 as an illustration of the determinations.

The original ground seeds. Some of the seeds (200 g) were removed from the shells. From a small sample, the brown seed coats were removed after dipping the seeds into boiling water for 45 sec; the seeds free from coats were tested. From the results of tests (rats 1 and 2, Table 1), it was concluded that there was no point in treating the seed coats and the remainder separately as the toxicity was in the seed proper.

Petroleum ether extract of ground seeds. The remaining 199 g of seeds were ground in a coffee mill and then extracted successively at room temperature with approximately 700 ml, 350 ml, and 350 ml of light petroleum (b.p. 40°–60 °C), each extraction being left overnight. The light yellow filtrates were combined, and the light petroleum removed by distillation. The residue weighed 14 g (the yield from the seeds may vary because on another occasion, 100 g of seeds ground more finely gave 13·5 g after completing the extraction with a Soxhlet).

The fatty residue was saponified for approximately 1 min with boiling methanol and KOH: (KOH 6 g, H_2O 4·05 ml, methanol 36 ml). The residue and alcoholic KOH were warmed separately, rapidly mixed and kept in the hot water bath. On cooling, the whole solidified. Ether (anaesthetic) was then added to a total volume of 460 ml.

The ether-insoluble precipitate (I) (separated by filtration) was treated with 5 ml of H_2O, 8 ml of 50 per cent (v/v) H_2SO_4, 50 ml of H_2O, and finally 50 ml of light petroleum (b.p. 40–60 °C). After shaking and standing, the petroleum layer was separated and washed three times with water to remove any free acid, after which the petroleum was removed by passing nitrogen through it at room temperature. The residue was 9·65 g and this was tested (rat 3, Table 1); 234 mg/kg were toxic.

The light brown fatty acid mixture was dissolved in acetone in a 50 ml centrifuge tube, and the precipitates at room temperature (18–20 °C), − 4 °C and − 20 °C

TABLE 1. TESTS ON RATS

No.	Weight (g)	Sex	Dose	Effect	Citric acid at death (μmole/g wet weight) Lung	Liver	Heart	Brain	Kidney
A selection from the numerous rat tests during the fractionation. Intraperitoneal injections unless stated otherwise									
1	175	♀	Ground seed coats (67 mg) oral.	Heart rate lowered from 480 to 380/min, normal next day.	—	—	—	—	—
2	200	♀	Seeds (500 mg) mixed 50/50 with rat cake (oral).	By next morning, animal semi-convulsive. Heart rate 192/min, on third day animal not well, heart rate 432/min, on fifth day, heart rate irregular 264/min, 10 a.m. convulsion.	—	—	3·93	2·56	0·06
3	175	♀	41 mg from pet. ether extract, in warmed arachis oil (0·15 ml)	63 min after injection, heart rate 108/min, 135 min convulsion, animal killed 210 min.	—	2·68	8·30	2·58	5·68
4	70	♂	2·8 mg from cold acetone supernatant emulsified in 0·1 ml 70% acetone, 2·8 mg more at 97 min. Total dose 80 mg/kg	Temporary drop in heart rate to 316/min then recovered, in 4 hr heart rate of 120, 5¾ hr killed when moribund.	3·85	1·50	9·16	2·25	3·41
5	180	♀	4·8 mg in 0·15 ml arachis oil from precipitate II	After 2 hr heart rate 240, 107 min convulsion, animal killed in hypersensitive state after 4¼ hr.	—	—	—	—	—
6	210	♀	25·7 mg in 0·1 ml arachis oil from chloroform–methanol extract.	After 56 min heart rate was 204/min and irregular, in 4 hr heart rate 120/min, in 6 hr 20 min rat killed	—	1·20	10·60	0·67	3·69
7	225	♀	13·0 mg ($CHCl_3$ methanol extract) in 0·1 ml arachis oil	After 1 hr heart rate 340/min, 3 hr 38 min heart rate 204/min, next day heart rate 336/min, third day heart rate 480/min, recovered	—	—	—	—	—
Toxic peak			Dose*						
8	165	♀	2·34 mg in 0·55 ml; 14 mg/kg	At 30 min heart rate 300, at 1¾ hr heart rate 96, sprawling, at 3 hr convulsion when handled, at 4½ hr died in tonic convulsion.	—	5·72	6·95	2·88	—
9	160	♀	1·56 mg in 0·23 ml, 9·4 mg/kg.	At ¾ hr heart rate 240 irregular, at 3¾ hr small convulsion, at 4 hr convulsion and at 5½ hr killed	—	5·9	1·36	0·32	2·68
10	200	♀	0·78 mg in 0·25 ml, 4·7 mg/kg.	Not much affected, at 1 hr heart rate dropped slightly (to 370) during the first 4 hr and then recovered.	—	—	—	—	—

* Three toxic peaks from the three runs of the columns combined and volume reduced to amounts shown for intraperitoneal injection. Injected solutions would contain small amounts of residual acetone which are known to be tolerated by rats.

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successively removed, leaving most of the toxicity in the acetone supernatant at — 20°C; 80 mg/kg was toxic to the heart (rat 4, Table 1).

The precipitates obtained were redissolved and reprecipitated at various low temperatures, the final supernatant being added to the above. It has been found consistently that, after such reprecipitation, toxic activity is to be found in the most soluble fraction; the precipitates which separate were proved by test to be relatively inactive.

The filtrate from precipitate (I) was treated with a further 200 ml of ether, which gave a further precipitate (II). After removal of the ether from the supernatant, addition of 100 ml of ether gave a further precipitate (III). The combined precipitates (II) and (III) were treated with H_2O (30 ml) and worked up by acidification and extraction with light petroleum as precipitate (I). The remaining oil weighed 1·735 g and was tested on rat 5 (see Table 1); 25·7 mg/kg was toxic.

Chloroform–methanol extraction of ground seeds. The seed powder after the initial extraction with light petroleum was treated in a Soxhlet extraction thimble with 1 : 1 chloroform–methanol. After removal of this, the fatty acid fraction weighed 17·0 g. (On the other occasion when there was a Soxhlet extraction of the first light petroleum soluble fraction, this latter weighed only 7·5 g showing that most of the fatty acid could be removed by intensive extraction with light petroleum only.) After treating with water, an ether extraction removed little; but the ether was washed and the washings added to the aqueous phase. The latter was acidified strongly with HCl (concentrated) and then extracted three times with light petroleum. The aqueous phase was separated; but the washing of the petroleum layer proved troublesome owing to the appearance of a gel between the phases. The petroleum layer, which was coloured, was tested on a rat (rat 6, Table 1), 122 mg/kg being toxic.

After removal of light petroleum from all the samples, the yellow oil remaining weighed 3·74 g. It was fractionated successively with acetone (10 ml) at — 4°C and — 20°C. A test on the — 20°C soluble fraction showed little activity (rat 7, Table 1), 58 mg/kg being toxic.

It can be concluded that the fatty acids specifically separated from the chloroform–methanol fraction have less total activity than the main fraction. No further work was done upon this fraction.

Final extraction with HCl

The residue from the chloroform–methanol extraction was washed with water (a procedure known not to remove activity), and extracted with N HCl (120 ml) at 80–90°C for 1–2 hr. The acid aqueous extract after removal by filtration *in vacuo* was neutralized to pH 7·0 with 95 ml 2 N NaOH and evaporated to form a sticky gum (65 g) which was shaken with successive samples of 80 per cent alcohol. Tests were made on the alcohol extracts with negative results.

In another experiment, we found that the acid extract of the residue from $CHCl_3$–methanol extraction was toxic, leaving then an inactive residue. As this acid extract might have contained any fluoroacetic acid present in the seeds, it was fractionated into alcohol, but no crystals of potassium fluoroacetate were obtained. Hence we could not repeat the observation recorded previously.[10]

Improved procedure

In the last fractionations done, the fatty fraction from 300 g of seeds after hydrolysis

Table 2. Fluorine partition of seeds of *Dichapetalum toxicarium*

	Amount (g)	F content (mg)	% reckoned as long chain fluoro fatty acid	Total fluoro fatty acid per 100 g seed
1. Original ground seeds	100	203·5	3·22	3·22
2. Light petroleum extract	13·5	167·5	19·5	2·64
3. Light petroleum residue	(86·5†)	42·1	0·76	0·66
4. $CHCl_3/CH_3OH$ extract	7·5	13·1	2·78	0·21
5. $CHCl_3/CH_3OH$ residue	(79†)	21·2		0·33
6. HCl extract	—	9·0	—	0·4
7. HCl residue	34	7·8	0·36	0·12
8. Total fatty acids from HCl extract*		5·50 = 61 % of HCl extract		

Note: 2 + 4 + 5 = 201·8 mg F (a figure close to that of the original 203·5 mg F).
† by difference. * after hydrolysis and extraction.

was acidified with HCl, and extracted directly in acid solution with light petroleum as the ether extraction at the alkaline stage seemed unnecessary. Following this the washed petroleum extract was fractionated as before in the cold. The final fraction at — 19°C contained about 16 g solid.

An approximate balance sheet for fluorine has been made using the thorium titration after fusion in the bomb (Table 2).

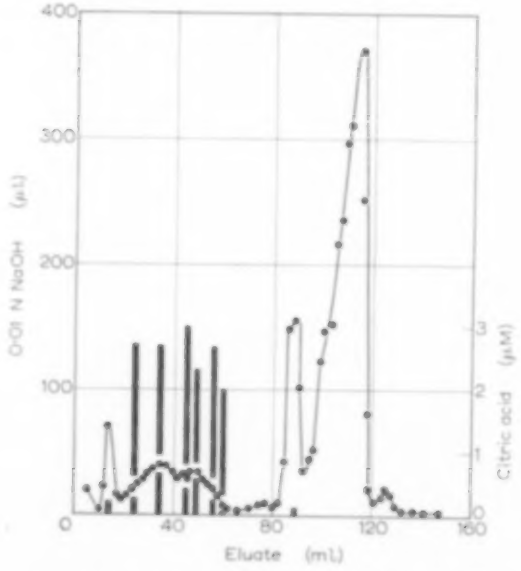

Fig. 1. Course of fractionation by column chromatography. Ordinates, µl NaOH in methanol for neutralizing acid in sample; 0·04 per cent phenol red was used as indicator. Abscissae, 2·0 ml samples. The toxic substance came out between 18 and 54 ml. Solid black lines indicate µmoles citric acid inhibited by comparable amounts in the standard kidney test. The peak at approximately 15 and 85 had little inhibitory activity and the high peak at about 110 showed none. Eluant 55 per cent acetone until 65 ml when it was changed to 60 per cent acetone.

This shows that, reckoning from the fluorine content, 82 per cent of the fluoro-fatty acid is extracted in the light petroleum extract and that in the further chloroform–methanol extract, there is only 6 per cent more. The fluorine content of the present samples is considerably higher than that found in the crude fat from the original seeds.[10] The reason for this is not known; but it could be due to natural variations. Differences in the content of alkaloids in plants are well recognized.

Chromatography. The fractions soluble in light petroleum at about 20°C were dissolved in minimal volumes of 70–80 per cent acetone (5 ml) and subjected to reversed-phase partition chromatography in amounts not exceeding 20–30 mg in dry weight.

Figure 1 is a confirmation with the fresh batch of seeds of the course of the previous fractionation.[10] Though care was taken to prepare the columns uniformly, it was rare for them to behave in exactly the same way. Nevertheless, the general pattern showed clearly that the active peak was followed by peaks containing far larger amounts of fatty acids which were not toxic and which did not contain fluorine. The tests were made on the citric acid metabolism of kidney particles *in vitro*, and also on rats (Table 1, rats 8, 9 and 10).

It was found best to combine and to refractionate the toxic peaks on columns of the same size. To prepare samples for this, similar fractions from about eight columns were combined and subjected to a stream of CO_2 free air until the volume was sufficiently reduced to a stage where the acetone had been removed (usually about 20–30 ml). Figure 2 shows the course of a refractionation. It was usual for the sharp

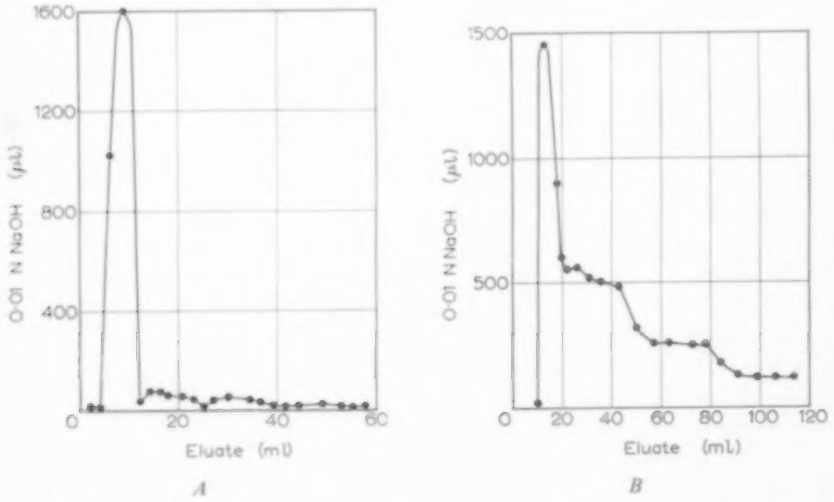

FIG. 2. *A* and *B* represent two refractionations of the toxic peaks. Ordinates, μl 0·01 N NaOH in methanol, 0·04 per cent phenol red as indicator. Abscissae, eluates (ml). Eluant 60 per cent acetone. *A* shows an example of the best type of refractionation, and *B* of a refractionation in which there is still some other unsaturated acid present which shows especially in the later stages of the fractionation.

rise to a peak to be followed by a less rapid fall. About 10 per cent of the fraction soluble in light petroleum at − 20°C may be expected in this peak.

Isolation of the active fraction as an oil. The acetone solution after titration with 0·01 N NaOH was subjected to a current of air at room temperature to remove all the acetone. At first this was done with N_2; but air was substituted for reasons of convenience, and did not appear to induce any loss of activity.

The final solution in a convenient volume was acidified with 6 N HCl in presence of a trace of phenol red to give a final concentration of 1 N HCl, and extracted with light petroleum (40°–60°C). After thorough washing, the light petroleum was removed and the product precipitated with calcium salts to free it from traces of paraffin and (possibly) long chain alcohols.

Calcium purification. The residue from the light petroleum (a few milligrams) was dissolved in the minimum volume of absolute acetone. The pH was brought to 8·0 (approximately) using 0·2 N NaOH and phenol red as indicator. One millilitre of $CaCl_2.6H_2O$ (20 per cent w/v) in 50 per cent (v/v) aqueous acetone made alkaline with 1 N NaOH to pH 7–8 was added to make total acetone concentration 75 per cent (v/v); the mixture was thoroughly shaken, pH adjusted to 7–8 by careful addition of 1 N NaOH and allowed to stand for 1 hr in the refrigerator at + 2·0°C. The precipitated Ca fatty acid salt was separated by centrifuging and washing twice with the acetone solution of $CaCl_2$ (1 ml), followed by two washings with 2 ml light petroleum to free from traces of paraffin. The precipitate was now treated with 2 N HCl (2 ml) and emulsified with the aid of a glass rod to convert it to the free fatty acid. After extraction with petroleum (b.p. 80–100°C) to reduce the presence of H_2O or acid in the final phase, the extract was washed twice with H_2O (2 ml), evaporated to dryness, and re-extracted with light petroleum (40–60°C) into a weighed tube. The oil was finally dried *in vacuo* over KOH.

Properties of the oil. The oil so obtained was almost colourless and melted at 11·5–14·5°C. It contained no P (less than 0·2 per cent) and no N (less than 0·5 per cent), also no S (testing for sulphate after boiling with HNO_3). Various specimens were examined for content of F, double bond C, as well as —COOH, and also by infra-red spectroscopy. If the compound was an ω-fluoro-octadecenoic acid ($FCH_2.(CH_2)_{7-n}$ $CH = CH(CH_2)_{7+n}COOH$), the m.w. should be 300, and the F content 6·33 per cent.

Infra-red observations. A specimen of very small amount was kindly examined by Mr. L. C. Thomas at Porton who reported: "The spectrum was very similar to that of oleic acid, with addition of a band in the region where C—F linkages absorb. There was some internal evidence from the C—H vibration that the F was in a terminal FCH_2 group. It was unlikely that the compound contained more than one double bond." Mr. Thomas was able to say definitely that there was no indication of a pentenyl ring as in chaulmoogric acid.

Fluorine content. The following estimations were made:

(1) Estimation made by the spectrochemical method by Mr. Birks at Harwell required investigation to get the best conditions. The earliest estimations on impure fractions ranged from 5·2–6·0 per cent; but they were vitiated by the presence of too much K, and by the difficulty of getting satisfactory carbon fluorine standards. He reported that in the last and most accurate estimation, a mixture of sodium fluoroacetate, oleic acid and bromoacetate was used as a standard. In this ten spectrograms were obtained from a sodium salt (equivalent to 1·766 mg), giving a value of 5·89 $\pm$ 0·46 per cent of F a result to be considered slightly low rather than high. Hence the value was consistent with the theoretical for a fluoro-octadecenoic acid of 6·33 per cent.

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(2) In the nine fluorine estimations done by thorium titration after combustion in the bombs on the toxic fractions, all fell between 5·4 and 6·1 per cent. Of these, the more reliable are given in Table 3; it will be noted that for fraction 1, the

TABLE 3. FLUORINE, DOUBLE BOND AND OTHER ESTIMATIONS ON FATTY ACID FRACTIONS*

Fraction	Total sample (mg)	F (%)	CH = CH	COOH m.w.	m.p. (°C)
1	42	6·04 6·03	1·00	305 ± 5	11·5–12·5
2	23	5·82	1·05†	301	13–14
3	62	5·42 5·62, 5·74 Av. 5·55	1·10	302·5	12–14·5
Theoretical for fluoro-octadecenoic acid		(6·33)	(1·00)	(300·0)	

* 10 Fluorodecanoic acid 0·459 mg gave fluorine 45·7 μg or 99·6 per cent theoretical. It was estimated in triplicate. Since the blank as determined was 0·2 μg, this made the corrected value 94·5 per cent theoretical.

† Triplicate.

double bond estimation is 1·00. The fluorine estimation averaged 6·035, i.e. 4·5 per cent lower than the theoretical for a (C_{18}) acid containing one double bond of 6·33. The value obtained by Mr. Birks on this same specimen was 5·89 ± 0·46 per cent. Taking into account the above considerations and that of the m.w. calculated by the —COOH titration, it appears that this was a virtually pure fluoro-octadecenoic acid.

In the case of the two other specimens obtained in other runs, the agreement was less good. As the m.w. was 300, the higher double bond of 1·01–1·10 indicates small contamination with some acid or acids containing more double bonds. It is notoriously difficult to separate acids of this type completely; thus Crombie *et al.*[3] found a tendency for linolenic acid (three double bonds) and even linoleic acid to run somewhere near the saturated C_{14} acid. The tailing of our repurified fractions on chromatography suggested the presence of some slower running components. If the contaminant was linolenic acid, a double bond of 1·06 would be given by 3 per cent contamination and of 1·10 by 5 per cent. Hence the F content would become 6·22 or 6·02 or allowing for the 4 per cent correction, 5·95 and 5·76. The value of 5·82 for the specimen 2 and of 5·55 for specimen 3, suggests the presence of a trace of linoleic acid too. At first we thought that traces of a C_{20} F containing acid might be present; but this now appears to be excluded.

The question arises whether there is toxicity or F in the specimens of fatty acids running in different fractions separated from the columns. In one run, 2·0 ml samples taken from sample no. 43 to the end contained no fluorine; in another, samples 38 to 42 contained no apparent fluorine but very slight toxicity. The evidence is therefore that there is present only one F containing fatty acid.

Nature of the substance. In favour of the view that we are dealing with a C_{18} fluoroacid with one double bond, there is the following evidence.

(1) Nitrogen, phosphorus and sulphur are not present.

(2) On the reversed-phase column, the main substance runs at about the same place as a saturated C_{13} acid: (*a*) acids with one double bond appear to run in the same place,[3] as a saturated acid with two C atoms less; (*b*) the fluoroacids tried, namely ω-fluorododecanoic and ω-fluorostearic acid ran as if they were saturated acids with three C atoms less.[10] Hence a C_{18} acid containing one double bond and one fluorine atom should run in approximately the same place as a saturated compound containing C_{13} which was found.

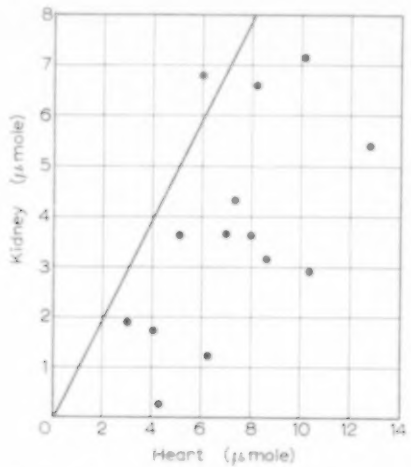

Fig. 3. Diagram shows the relation between citric acid in heart and kidney for rats tested with the fluoro fatty acids. A straight line indicates the ratio 1 : 1. It will be seen that most ratios exceed 1 lying on the right-hand side of the line.

(3) The —COOH titration gave approximately 300 for the molecular weight.

(4) The estimation of —CH = CH— gave in one case one double bond per one —COOH and in other cases a 5 per cent lower result which is explained by contamination with linolenic acid.

Table 4. Inhibition of citric acid metabolism in kidney particles by purified fluoro-fatty acid

	Citric acid/flask (μmole)	Difference
Control	0·84	
+5 μg F acid	6·80	5·96
2·5 μg F acid	5·66	4·82
1·25 μg F acid	3·60	2·76

Each flask contained: enzyme (1·9 ml in 1·0% KCl with phosphate buffer 0·1 M KH_2PO_4 neutralized with NaOH, pH 7·2); $MgCl_2.6H_2O$ (0·8% w/v in 0·1 ml); Na_2 ATP (0·33 mg in 0·1 ml); Na fumarate (3·2 mg in 0·2 ml); H_2O (0·2 ml); addition of either 0·05 ml 50% alcohol or 0·05 ml containing fatty acid dissolved in 50% alcohol; KCl 1% w/v to 3·0 ml. Incubated for 40 min at 38° C then boiled, centrifuged and 1 ml removed for estimation of citric acid.

The presence of a substance resembling fluorocitric acid in its action was established qualitatively by tests upon brain particles.

(5) The F determination approximated to the theoretical of 6·33 per cent, but tended to be about 4 per cent too low.

(6) The m.p. is close to that of oleic acid (13·0°C ± 1·5 instead of 14·0°C).

(7) The ratio of citric acid heart/kidney (see Fig. 3) is like that of long chain ω-fluoro-fatty acids tried.

Biochemical and biological activity of purest samples

Biochemical activity. One specimen of the highly purified acid, dissolved in 50 per cent (v/v) alcohol, has been tested for inhibitory activity with kidney particles (Table 4). Even as little as 1·25 μg acid per 3 ml ($4{\cdot}2 \times 10^{-3}$ μmole) inhibited the disappearance of 2·76 μmoles of citric acid in our standard test. In this respect, it was about twice as active in terms of the FC component as our enzymically prepared fluorocitric acid of which 0·6 μg will inhibit 1 μmole citric acid.[9] The reason for this difference is not known yet, but it suggests that the intramitochondrial conversion of fluoro-octadecenoic acid to fluorocitric acid is very efficient. This confirms a previous conclusion for fluorodecanoic acid.[10]

Biological activity. Typical biological activity and citric acid estimations at death for rats obtained during the course of the fractionation have been shown above in Table 2. A final two tests were done on the calcium purified substance and are shown in Table 5. From these (Table 5) and the previous tests of the less pure peaks (Table 2), it may be roughly estimated that the LD_{100} appears to lie between 5 and 7 mg/kg.

TABLE 5. TOXICITY TESTS UPON PUREST FLUORO-FATTY ACID

Specimen 1 of the Ca purified fatty acid was injected into two small rats and was found to be toxic in doses of 9·0 and 7·1 mg/kg, respectively.

(1) Rat 80 g ♂ intraperitoneal dose of 9·0 mg/kg in 0·1 ml arachis oil. After 30 min heart rate 480. At 45 min 430 beats, missing. After 1 hr 10 min the heart was too irregular to count. At 2 hr 35 min a minor convulsion and died at 2 hr 55 min. Citric acid content μg/g heart 1175, kidney 1806, liver 390, brain 647, lung 620.

(2) Rat 90 g ♂ intraperitoneal dose of 7·1 mg/kg in 0·05 arachis oil + 0·075 ml alcohol. After 30 min heart rate 480. At 45 min 440; at 1 hr 10 min 460. Died at 2 hr. 10 min.

DISCUSSION

The toxic component appears to be an unsaturated acid containing one fluorine and eighteen carbon atoms. It was not always possible to free it from traces of other C_{18} acids containing more than one double bond; there is no evidence that other fluoroacids were present, an unusual feature in the field of seed fats where a mixture of fatty acids are usually found. We are endeavouring to improve the method of extraction so that enough fatty acid can be obtained to determine the position of the FC grouping and the double bond, and to settle the LD characteristics.

There are four main points of general interest: (a) that another compound with a FC grouping is found in nature in another species of the same genus as the South African *Dichapetalum cymosum*; (b) that the FC fragment which starts presumably as a C_2 compound is found in the seeds as a fluoro-octadecenoic acid, why is the F not found in the large amount of other fatty acids separated? (c) the problem is raised again as to how the plant synthesizes the FC bond, and (d) as to whether the heart or kidney enzymes concerned in the breakdown of the long-chain fatty acid and con-

version of this to fluoroacetyl-CoA and fluorocitric acid are the same as those degrading ordinary C_{18} acids.

These questions are for future work. In so far as the heart gives a higher citrate accumulation than the kidney after intraperitoneal injection of the fluoro-fatty acid, it would appear that the enzymes in heart tissue must be efficient at metabolizing the fluoroacid, and suggests that heart tissue utilizes fatty acids *in vivo* more readily than has often been recognized.

Acknowledgements—In addition to those mentioned in the text, viz. Mr. L. C. Thomas and Mr. F. T. Birks, we are grateful to Dr. F. L. M. Pattison (London, Ontario) for gifts of the synthetic fluoro-fatty acids, to Dr. T. Moore and Dr. Rhodes for valuable advice and to the latter for a double bond estimation; also to Dr. R. M. C. Dawson, Dr. V. P. Whittaker and to others on the staff of the Biochemistry Department for advice; also to Miss R. Shawdon for skilful technical assistance.

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THE EFFECTS OF VARIOUS POISONS ON THE GROWTH AND VIABILITY OF SPORELINGS OF THE RED ALGA *PLUMARIA ELEGANS* (BONNEM.) SCHM.

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Abstract—The heavy metals mercury, silver, copper and arsenic are more toxic than the non-metallic inhibitors hydrocyanic, hydrazoic, iodoacetic, fluoroacetic and malonic acids and 2 : 4-dinitrophenol to sporelings of *Plumaria elegans* in sea water at pH 8·1. Mercury and silver are more toxic than copper and arsenic and the toxicities of mercury and arsenic are greatly increased when these poisons are used in the form of organic compounds. Thus methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *iso*propyl-, *iso*amyl- and phenylmercuric chlorides and phenylmercuric iodide are more toxic than mercuric chloride; phenarsazine chloride is more toxic than arsenite.

The toxicities of organic derivatives of mercury and arsenic are less when the poisons are used in the presence of an excess of reduced glutathione, and the thiol compound also markedly reduces the toxicities of copper, silver and arsenite. When sporelings that have been immersed in toxic solutions of all the poisons are subsequently washed with reduced glutathione, toxic effects are diminished.

The toxicities of an homologous series of primary *n*-alkylmercuric chlorides increase to a maximum with that of *n*-C_3H_7HgCl. Lipoid solubilities based on methyl-oleate: sea-water partition coefficients correlate with appropriate toxicity data for the first three members of the series. However, those of *n*-C_4H_9HgCl and *n*-$C_5H_{11}HgCl$ increase without any corresponding rise in toxicity and the lipoid solubility of phenylmercuric chloride is also higher than would be expected from the toxicity of this compound. The "cut-off" in relative toxicities within the homologous series is not found in complementary studies using crustaceans.

Mercuric iodide is far more toxic than mercuric chloride both to crustaceans and to *Plumaria*, but whereas various organic derivatives of mercury become markedly more toxic to crustaceans when used in the presence of excess KI, their toxicities to *Plumaria* are not increased.

The results have been discussed with special reference to various hypotheses concerning the modes of action of heavy metals as poisons to plants and to crustaceans.

INTRODUCTION

It has long been known that certain heavy metals are very efficient as poisons to various marine crustaceans, and more recent evidence suggests that this is because the poisons can readily penetrate into the test animals.[1, 2, 3] In the present work the toxicities of mercury, silver, copper and arsenic have been compared with those of several non-metallic inhibitors of respiration, using sporelings of a marine red alga as the test material.

The results again indicate the importance of penetration as a factor influencing the toxicities of heavy metals, and are also consistent with the view that sulphydryl groups are involved in the mechanisms of toxic action of these poisons. In addition, at least

one important difference has been found between the toxicological behaviour of a series of poisons to crustaceans on the one hand, and to a marine plant on the other.

GENERAL METHODS

Test material

The plant used in the present work was the red alga *Plumaria elegans* (Bonnem.) Schm. As it occurs in large quantities and has a protracted period of fruiting, this plant is very suitable material for use in experimental work on the early growth of algal sporelings.

After they had been collected from an intertidal zone the plants were packed in polythene bags and taken at once to the laboratory where they were kept under circulating sea-water until needed. It was found that material treated in this way remained healthy for several weeks but, for the purposes of the present work, settlement of spores was always induced within 7 days of the collection of the plants. To bring this about, fruiting branches of *Plumaria* were laid on glass slides placed in an enamelled dish containing filtered sea-water and left overnight. The next day the branches were removed and the spores that had been liberated were left undisturbed on the slides for a further 2 days. This latter precaution assured firm attachment of the sporelings to the slides so that they would not be removed by subsequent experimental treatments. Usually sufficient plant material was used to yield approximately 300 sporelings on each slide.

For the purposes of comparison a few experiments in the present study were made with larvae of the brine shrimp *Artemia salina* (L)., adults of the copepod *Acartia clausi* (Giesbrecht) and nauplii of the barnacle *Elminius modestus* (Darwin). Details of how these three kinds of test material may be obtained have been given earlier.[1, 2]

Poisons

Mercury. The organic compounds of mercury used in this study were methyl-, ethyl-, *n*-propyl-, *n*-butyl-, and *n*-amylmercuric chlorides (hereafter referred to as I-V), *iso*propyl- and *iso*amylmercuric chlorides (VI and VII), phenylmercuric chloride (VIII) and phenylmercuric iodide (IX). The method used to dissolve these compounds in sea-water was that described by Corner and Sparrow.[2] Inorganic mercury was used as mercuric chloride and as mercuric iodide.

Copper. Copper sulphate ($CuSO_4.5H_2O$) was added to sea-water in an amount equivalent to 1 mg Cu/l. This concentration of copper is not very toxic to *Plumaria* but higher concentrations of the metal could not be used because they form unstable solutions in sea-water. Accordingly, very few experiments were done with copper as the poison.

Silver. Before any toxicity experiments could be made with silver it was necessary to determine the quantity of the metal that could be dissolved in sea-water. To do this use was made of tracer isotopes. Radioactive silver wire (^{110}Ag) was obtained from the AERE, Harwell, and the material (1 g) was left overnight to dissolve in 5 N HNO_3 (20 ml). A known volume (1 ml) of the stock solution so formed was added to sea-water (1 l.) and the mixture was stood at room temperature for 1 hr. After this time, the greyish-white precipitate had coagulated and was removed by filtration on a Whatman no. 42 filter paper. The filtrate was then adjusted to pH 8·1 with 0·5 N NaOH and the quantity of silver present in solution was estimated from measurements of the

radioactivity in 0·1 ml samples. It was found that the solution contained 2·01 mg Ag/l.

Arsenic. The organic compound of arsenic used in this study was phenarsazine chloride. To prepare a solution of this substance in sea-water, 0·05 g of the poison was wetted with a few drops of ethanol, and the paste so formed was added to sea-water (1 l.) contained in a darkened bottle. The mixture was shaken continuously for 24 hr, after which time the material that had not dissolved was removed by filtration. Under these conditions the amounts of phenarsazine chloride that dissolve in sea-water have been determined many times and found to be equivalent to 0·35–0·70 mg As/l. (R. Robinson, private communication). In all experiments with organic compounds of arsenic the solutions were kept in the dark because otherwise they lose their toxicities very rapidly.

Arsenic was also used as sodium arsenite. To prepare a solution of this poison in sea-water, a known quantity of arsenious oxide was dissolved in 5 N NaOH and a measured volume of the solution was added to sea-water which was then adjusted to pH 8·1 with 0·1 N HCl.

Non-metallic poisons. Other poisons used were 2 : 4-dinitrophenol and iodoacetic, fluoroacetic, malonic, hydrazoic and hydrocyanic acids. Each of these compounds is well known as an inhibitor of intermediary metabolism. All the poisons dissolve readily in sea-water.

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Toxicity experiments

Plumaria. Glass slides to which sporelings had become attached were immersed for measured times in the various toxic solutions under test, then removed and washed free from the poison with plain sea-water, transferred to an enamelled dish and covered with filtered Plymouth "outside" sea-water. The sporelings were kept in the dish for 7 days at room temperature near a north window, and under these conditions the material used in control experiments showed healthy, rapid growth. In early experiments attempts were made to increase the rate of growth by adding various nutrients to the medium. However, this led to the development of heavy crops of diatoms that obscured the *Plumaria* sporelings, and was therefore discontinued. At the end of 7 days, each slide was placed in a petri dish, covered with a film of sea-water and examined through a binocular microscope (magnification × 100). A number of traverses, each of which covered several "fields", was made across each slide, and the conditions and stages of development of the sporelings in every field observed. Typically, some 40 "fields" were examined and the total number of sporelings in them was about 300. Dead sporelings were easily recognized because the contents of the cells had markedly contracted and the colour of the cells had changed from red to pink or green. Quantitative measurements of the extents of development were facilitated by the well-defined sequence of growth shown by the sporelings, a sequence typical of the Ceramiales and distinguished from that of the other Florideophycidae by early formation of a rhizoid and a filament of cells. Usually it was found that from two to three cells had formed during the 2 days allowed for settlement and that within the next 5 days a filament developed containing from four to five cells. Any sporeling that had not grown beyond the two to three cell stage was described as "undeveloped", in that no apical cell had been formed and, therefore, elongation of the filament had not begun. It was at this "undeveloped" stage that the sporelings

were immersed in the toxic solutions and it was frequently found that the poisons, although they did not kill the sporelings, prevented any further development.

Postponement of examination of the sporelings until 7 days after they had been immersed in the toxic solutions was not an arbitrary choice. Experiments with $HgCl_2$ showed that after the sporelings had been immersed in various concentrations of this poison and then transferred to fresh sea-water, they continued to die for several days, and, in fact, percentage mortality did not attain a maximum before 5 days had elapsed (Fig. 1). Accordingly, sufficient time (7 days) had to be allowed for maximal toxic effects to beome manifest.

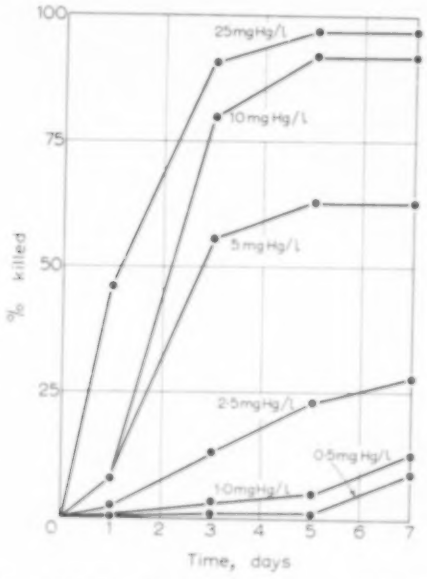

Fig. 1. Cumulative toxic effects of $HgCl_2$ to *Plumaria* sporelings initially immersed 0·5 hr in sea-water containing different concentrations of the poison, then transferred to fresh sea-water and examined at various subsequent times.

Crustaceans. The methods used in toxicity tests with *Elminius*, *Acartia* and *Artemia* were those described in earlier studies.[1, 2] However, an additional series of experiments was made with *Elminius* in which the procedure used was similar to that just described for studies with *Plumaria*. The test animals were immersed in the toxic media for 30 min, then strained off on bolting-silk (200 mesh), washed with plain sea-water and transferred to filtered sea-water. After 18 hr, and again after 42 hr, counts were made of the number that had died.

Partition coefficients

In a previous study[2] the partition coefficients of various organomercury compounds between ethyl ether and sea-water were determined in order to estimate the lipoid solubilities of these poisons. In the present work methyloleate was used instead of ethyl ether because it was thought advisable to employ a lipoid solvent having some relationship to fatty substances of plant origin.

Mercury. In order to determine the partition coefficients of organomercury compounds between methyloleate and sea-water use was made of the following analytical procedures. Organic mercury was first converted into inorganic by the method of Laug and Nelson[4] and then estimated by titration with chloroform-dithizone essentially as described by Harris.[5] The total quantity of the standardized chloroform–dithizone solution added gave a direct measure of the quantity of mercury that remained in the sea-water phase (200 ml) after the solution of the organomercury compound had been extracted with methyloleate (20 ml). From this value it was then possible to calculate the quantity of the organomercury compound that had been extracted into the fat, and this latter value, when divided by the concentration of the poison that was left in the aqueous phase, gave the partition coefficient.

Because the lipoid solubilities of the organomercury compounds were expressed in terms of that of mercuric chloride as a standard poison, it was necessary that a very accurate value should be obtained for the methyloleate:sea-water partition coefficient of this compound. To do this, use was made of $HgCl_2$ containing ^{203}Hg, and the methods employed were those described by Corner and Rigler.[3, 6]

Silver. In order to determine the appropriate value for AgCl use was made of ^{110}Ag.

RESULTS

Comparison of toxicities of heavy metals with those of non-metallic metabolic poisons

The findings shown in Table 1 demonstrate that the toxicities of various inhibitors of intermediary metabolism are much less than those of heavy metals to sporelings of *Plumaria* in sea-water at pH 8·1. Thus, with the exception of cyanide and fluoroacetate, these inhibitors have little influence on the development of the sporelings, whereas much lower concentrations of mercury and silver kill all, or nearly all, the test material, and copper and arsenic greatly retard its development.

Further experiments showed that all the poisons became much more toxic under conditions of lowered pH. For example, in contrast to the results obtained at pH 8·1, at pH 4·5 none of the sporelings survived immersion for 18 hr in fluoroacetate,

TABLE 1. EFFECTS OF VARIOUS POISONS ON THE GROWTH AND VIABILITY OF SPORELINGS OF *Plumaria elegans*

Poison	Concentrations (mg/l.)	State of sporelings after 1 week	
		% dead	% developed
Copper	1·0	3	11
Silver	1·0	98	0
Mercury	1·0	100	0
Arsenic	1·0	0	18
Malonate	50	0	69
Fluoroacetate	50	3	53
Iodoacetate	50	0	75
DNP	50	1	68
Azide	10	0	68
Cyanide	10	0	33
Control	—	0	77

Sporelings (ca. 300) immersed 18 hr in toxic media. Copper used as $CuSO_4 . 5H_2O$; silver as $AgNO_3$; mercury as $HgCl_2$ and arsenic as sodium arsenite: malonic, iodoacetic, fluoroacetic, hydrazoic and hydrocyanic acids used as sodium salts: DNP = 2 : 4-dinitrophenol.

iodoacetate, 2 : 4-dinitrophenol and azide:malonate and cyanide also killed a significant number.

Data comparing the toxicities of the poisons to *Plumaria* in the light and in the dark were also obtained. However, with the exception of copper, which is more toxic in the dark, no significant differences were found.

Use of heavy metals as their lipophilic, organic derivatives

The results of experiments in which organomercury compounds were used are shown in Table 2 and demonstrate that, compared with mercuric chloride, these poisons are highly toxic. There is also evidence that as the homologous series of primary *n*-alkylmercuric chlorides is ascended toxicity increases and that a compound with branched side-chain, as exemplified by VI, is less toxic than the corresponding

Table 2. Relative toxicities of organic and inorganic compounds of mercury

Poison	LD_{50} (mg Hg/l.)
I	0·044
II	0·026
III	0·013
IV	0·013
V	0·013
VI	0·028
VII	0·019
VIII	0·054
IX	0·104
HgI_2	0·156
$HgCl_2$	3·12

Sporelings immersed 18 hr in toxic media.

Table 3. Lowered toxic effects of poisons used in the presence of reduced glutathione

Poison	Conc. (mg metal/l.)	Time of immersion (hr)	State of sporelings after 1 week				Protection by GSH (%)
			% Dead		% Developed		
			SW	GSH	SW	GSH	
III	0·04	0·5	7	0	19	66	100
IV	0·04	0·5	78	36	1	69	54
V	0·04	0·5	86	26	0	65	70
VII	0·04	0·5	96	0	0	72	100
VIII	0·04	0·5	8	0	2	59	100
$HgCl_2$	0·2	18	52	18	2	52	65
Copper	1·0	24	43	0	0	45	100
Silver	0·2	18	67	0	0	56	100
Phenarsazine chloride	0·004	18	100	0	0	54	100
Control	—	—	0	0	79	74	—

Toxic media prepared in sea-water (SW) and sea-water containing reduced glutathione (GSH) in twentyfold excess of the poison; % protection estimated as

$$\frac{\%\ \text{dead (SW)} - \%\ \text{dead (GSH)}}{\%\ \text{dead (SW)}} \times 100\%.$$

straight-chain isomer. In addition, the toxicities of the aromatic poisons, VIII and IX are of the same order as those of *n*-alkylmercuric chlorides with short side-chains (II–III). Finally, it is interesting to note that mercuric iodide is more toxic than mercuric chloride.

Effect of reduced glutathione on the toxicities of heavy metals

Table 3 describes the results of experiments in which reduced glutathione was used to protect *Plumaria* from the toxic effects of heavy metals. As only a small proportion of the test material was killed in solutions of III and VIII, the influence of reduced glutathione on the toxicities of these two compounds is probably better estimated from the extents to which it reduced their abilities to inhibit growth; and an examination of the relevant data in Table 3 shows that, in this respect, the thiol compound is effective. The results of experiments with IV and V showed that the thiol compound was also effective in protecting the test material from the toxic effects of these poisons, for it markedly reduced the numbers of sporelings they killed. It was found that the thiol compound reduced the toxicity of mercuric chloride and had an even more marked effect on those of copper, silver and phenarsazine chloride.

TABLE 4. EFFECT OF WASHING POISONED SPORELINGS WITH REDUCED GLUTATHIONE

Time of washing with GSH (min)		0	3	6	9	12
Poison	Time of immersion (hr)	% dead after 1 week				
Copper	24	72	71	61	58	56
Silver	2	56	55	52	42	37
Mercury	2	27	15	11	6	4
IV	0·5	50	18	13	8	6
V	0·5	50	20	17	8	5
Phenarsazine chloride	0·5	100	69	47	37	33

Toxic media contained 1 mg copper, silver and mercury (as $HgCl_2$)/l.; 0·016 mg Hg/l. as IV and V and 0·04 mg As/l. as phenarsazine chloride. Washing medium contained 10 mg reduced glutathione (GSH)/l.

The results outlined in Table 4 provide data on the removal of heavy metals from the sporelings by reduced glutathione after poisoning had taken place. Thus, when sporelings that had previously been immersed in toxic solutions of copper, silver, mercury (as mercuric chloride) and phenarsazine chloride were subsequently washed with reduced glutathione for progressively longer times there was a continuous increase in the extent to which the toxic effects of the poisons were reduced. It will also be seen from Table 4 that treatment with the thiol compound reduced the toxic effects of IV and V when these poisons were used at low concentrations.

Differences between the toxicological behaviour of heavy metals to plants and to crustaceans

In Table 5 toxicity data obtained in earlier experiments with each of several species of crustacean are compared with the results of the present study using *Plumaria*. It will be seen that, of the three sets of results obtained in studies with crustaceans, it is

Table 5. Relative toxicities of mercury compounds to *Plumaria* and crustaceans

Poison	No. of times as toxic as $HgCl_2$ to			
	Acartia clausi	*Elminius modestus*	*Artemia salina*	*Plumaria elegans*
I	2·1	5·0	15	71
II	2·0	6·6	17	138
III	3·1	9·6	54	235
IV	2·7	17	294	240
V	3·8	21	980	240
VI	—	—	25	112
VIII	2·4	6·3	23	60
HgI_2	1·7	—	24	—

that for *Artemia* which approximates the most closely to the results of experiments with *Plumaria*. However, the two series of results showed some marked differences. Thus, whereas the toxicities of I, II, III and VIII relative to that of $HgCl_2$ are much greater in experiments with *Plumaria* than in corresponding experiments with *Artemia*, the reverse is the case when studies are made with IV and V. It seemed possible that this sharp "cut-off" observed in experiments with *Plumaria* might be a temperature effect. However, when the experiment was repeated at 5°C instead of 15°C, the "cut-off" was even more marked in that the values reached a peak with III and then diminished slightly with IV and V. The next possibility to be examined was that the

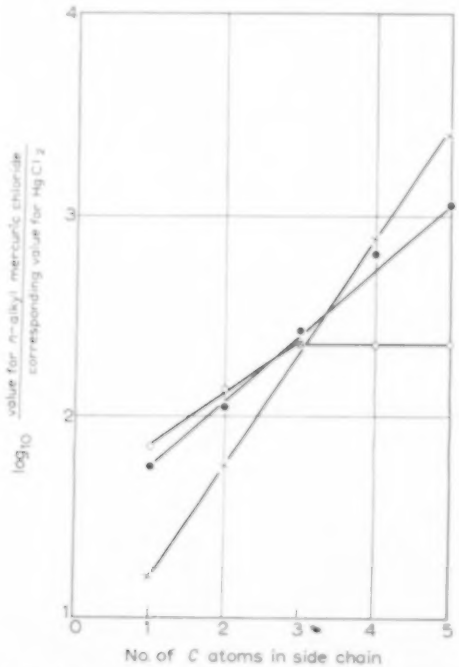

Fig. 2. Physical and toxicological data for primary *n*-alkylmercuric chlorides. O—O, toxicities to *Plumaria*; X—X, ethyl ether : sea-water partition coefficients; ●—●, methyloleate : sea-water partition coefficients.

"cut-off" in the relative toxicities of the organomercury compounds might to some extent reflect corresponding changes in their lipoid solubilities. Previous measurements of these values had been based on the partition coefficients of the poisons between ethyl ether and sea-water, and the results obtained showed a reasonable degree of correlation with the relative toxicities of the compounds to *Artemia*.[2] However, it seemed possible that the relative toxicities of the poisons to *Plumaria* would be more likely to reflect the partition coefficients of the poisons between sea-water and an organic phase more closely related to a plant oil.

Relevant physical data, expressed in a manner similar to that of Ferguson,[7] is included in Fig. 2 which demonstrates that the logarithms of the relative lipoid solubilities of the homologous series of *n*-alkylmercuric chlorides show a linear relationship whether the values are based on partition coefficients of the compounds between ethyl ether and sea-water, or on those between methyloleate and sea-water. However, the two sets of values lie along lines whose slopes differ markedly and it is that which joins the methyloleate : sea-water series of values that more closely parallels the line constructed from the results of toxicity experiments with *Plumaria*.

Further toxicity tests using III *and* V. The "cut-off" in relative toxicities to *Plumaria* seemed to exemplify a fundamental difference between the toxicity data obtained in experiments with an alga and those from earlier studies using various species of crustacean. Accordingly, it was necessary to ensure that this "cut-off" was a genuine phenomenon, and not simply the result of some inadequacy in the method of carrying out toxicity tests with *Plumaria*. Reappraisal of the techniques used in these tests indicated an intrinsic feature that might give rise to anomalous results. This was that the test material was not examined for signs of death or arrested development

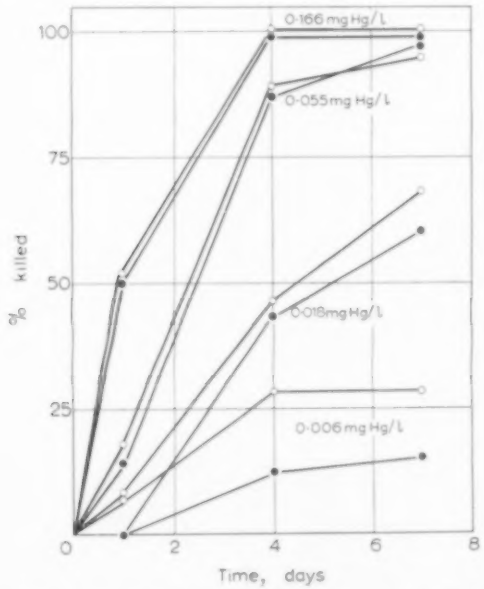

FIG. 3. Cumulative toxic effects of *n*-C_3H_7HgCl (●—●) and *n*-$C_6H_{11}HgCl$ (O—O) to *Plumaria* sporelings initially immersed 0·5 hr in sea water containing different concentrations of either poison, then transferred to fresh sea-water and examined at various subsequent times.

until 1 week after its immersion in the poisons. Therefore, more comprehensive toxicity tests were carried out in which the poisoned sporelings were examined several times during the week after their preliminary immersion in the toxic media, and equitoxic concentrations of III and V were determined after each of these experimental periods (1, 4 and 7 days).

The data obtained in these experiments are shown in Fig. 3 and demonstrate that, except at very low concentrations of the poisons, the curves obtained using III and V are almost identical. From these curves it was possible to determine the concentrations of the compounds that caused 50 per cent of the sporelings to die within 1, 4 and 7 days of their immersion in the toxic media. These values were then used to calculate the toxicities of the two compounds relative to that of $HgCl_2$, corresponding data for the latter compound being obtained concurrently.

Table 6

No. of days after immersion in poison	No. of times as toxic as $HgCl_2$	
	n-C_3H_7HgCl	n-$C_5H_{11}HgCl$
1	156	178
4	198	200
7	260	260

The results of these experiments are shown in Table 6 and indicate that the relative toxicities of both organomercury compounds, when estimated at progressively longer times after the sporelings were poisoned, show a steady increase. However, in spite of this, no marked increase is observed in the difference between the relative toxicities of the two compounds and the "cut-off" in relative toxicities is still as marked as that observed in previous experiments in which observations of toxic effects were made only on the seventh day after poisoning.

Changes in the toxicities of various mercury compounds induced by excess KI. The results, hitherto unpublished, of a previous study in which *Artemia salina* was used showed that the toxicities of various compounds of mercury to this animal were markedly increased when an excess of KI was added to the toxic media. These results are summarized in Table 7.

Table 7. Influence of KI on toxicities of mercury compounds to *Artemia* and *Plumaria*

Poison	% Decrease in LD_{50} (i.e. % increase in toxicity) induced by excess KI	
	Artemia	*Plumaria*
I	60	3
II	64	3
III	57	0
IV	58	0
V	4	0
VI	32	2
VIII	0	2
IX	0	5
HgI_2	43	0

KI used in tenfold excess of each posion.

The quantities of KI used in these experiments had no detectable toxic effects on the test animals yet considerably enhanced the toxicities of most of the poisons examined, including HgI_2. Exceptions were V, VIII and IX. Because experiments described earlier showed that there were certain marked differences between the results obtained when the toxicities of organomercury compounds were estimated using crustaceans and *Plumaria* as the test organisms it seemed worth while to examine the influence, if any, of KI on the toxicities of various compounds of mercury to *Plumaria*.

The results of these experiments (included in Table 7) demonstrate that there was no significant increase in the toxicity of any of the poisons examined, and this finding, therefore, provided evidence of a further difference between the toxicity data obtained using a crustacean and a marine alga as the test organism.

DISCUSSION

Results obtained in the present work have shown that, in general, as the lipoid solubilities of mercury compounds increase, so also do their toxicities to *Plumaria*. These findings emphasize the importance of penetration as a factor influencing the modes of action of mercury poisons: for, since the work of Collander and Bärlund[8] using *Chara ceratophylla*, it has become generally accepted that non-electrolytes penetrate the plant cell much more rapidly if they are highly lipoid-soluble. Peters, Sinclair and Thompson[9] associate the rapid rates (compared with arsenite) of penetration into human skin shown by certain organic arsenicals with the high lipoid solubilities of these compounds, and it therefore seems likely that the considerable toxicity of phenarsazine chloride to *Plumaria* is because this compound rapidly penetrates into the plant cell.

Further experiments have shown that reduced glutathione effectively lowers the toxicities of various heavy metals to *Plumaria* and that, when sporelings of the plant that have been treated with these poisons are subsequently washed with the thiol compound, cumulative toxic effects are reduced. These studies were of a nature similar to those carried out by various workers who have investigated the effects of heavy metals as enzyme poisons[9, 10, 11] and the results are consistent with the view that sulphydryl groups are in some way concerned in the mechanisms by which heavy metals exert their toxic effects to *Plumaria*.

Certain organic compounds of mercury are exceptional in that their lipoid solubilities are far higher than would be expected from their toxicities to *Plumaria*. These include *n*-butyl- and *n*-amyl-mercuric chlorides, the anomalous behaviour of which raises certain questions concerning the interpretation of toxicity data obtained in experiments with an homologous series. Many studies have been made of the variation in biological activity within an homologous series of poisons, and of the correlation that exists between the toxicities of such compounds and their physical properties. A comprehensive review of this subject has been given by Ferguson[7] and therefore it is not proposed to provide a detailed treatment here. However, it is worth mentioning that when homologous series of different poisons are tested for relative toxicities to several organisms, the number of carbon atoms at which maximum toxicity occurs is very variable. Thus Sexton[12] lists eleven such series in which the number of carbon atoms in the side chain of the compound that causes maximal toxicity varies between 1 and 14.

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More recently Ross and Ludwig[13] have reported that the fungi-toxicities of N-*n*-alkylethylenethioureas increase with increasing chain length up to and including the octyl homologue and then show a sharp decline with higher members of the series. These authors follow Sexton in attributing this "cut-off" in toxicity to the fact that, with higher homologues, it is water insolubility not lipoid solubility that determines toxicity. Ross and Ludwig claim that, as ethylenethioureas must be in solution before a partition can occur between the biophase and the surrounding medium, the amounts of the higher homologues available to the cell are limited by the low solubilities of these compounds in the aqueous phase. Such an argument, however, cannot be used in order to explain the "cut-off" observed in the relative toxicities of primary *n*-alkylmercuric chlorides to *Plumaria* because the solubility of the highest homologue (n-$C_5H_{11}HgCl$) in sea-water is far greater than that needed to provide a concentration equivalent to the LD_{50} for *Plumaria* (0·013 mg Hg/l.). Moreover, when the relative toxicities of the same series of compounds have been estimated using three species of crustaceans as test animals, it has been found that, although the resistances of the three species vary enormously, the values obtained continue to increase steadily throughout the series (see Table 5).

It is possible that at least two properties of a poison influence its ability to penetrate through a plant membrane. One is its oil–water partition coefficient, which is important in determining the absorption of the poison into the lipoid moiety of the membrane; the other is the diffusion coefficient, which affects the rate of passage of the poison through the lipoid layer. Although it has been found that the oil–water partition coefficients of certain *n*-alkylmercuric chlorides increase with increasing length of side-chain, no information is available concerning the corresponding diffusion coefficients of these compounds. However, it is possible that these values would diminish with increasing length of side-chain, not because of increasing molecular weight (which would have an insignificant effect) but because, as the numbers of carbon atoms in their side-chains increase, aliphatic compounds show a greater readiness to aggregate into micelles which would undoubtedly diffuse at a rate slower than that of individual molecules.[14] Therefore, one explanation of the "cut-off" in relative toxicities is that a steady state is reached which represents a compromise between absorption and diffusion. It seems possible that the point within the homologous series at which the steady state is reached would depend on the chemical composition of the lipoid barrier as well as on that of the poison and this may account for why the "cut-off" in relative toxicities has not been observed in experiments with *Artemia* and other crustacean species.

Further studies have shown that 2 : 4-dinitrophenol and iodoacetic, fluoroacetic, hydrazoic, hydrocyanic and malonic acids, compounds well known for their toxic effects on respiration, are far less effective than heavy metals as poisons to *Plumaria*. Moreover, the heavy metals are also more toxic than the other poisons to plants kept in the dark, a circumstance in which respiratory processes would become more important and the non-metallic inhibitors might be expected to exert greater toxic effects. These latter poisons did become far more toxic when the pH of the surrounding medium was lowered from 8·1 to 4·5, a change that would suppress their ionization and enable them to penetrate more readily into the plant cell; but even under these conditions they were still not as toxic as heavy metals, which also showed a marked increase in toxicity when the medium in which they were used was made more acid.

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One explanation of these findings is that heavy metals are far less specific than the other poisons in their toxic action; and this property, together with the well-developed intrinsic powers of heavy metals as enzyme poisons and their ability to penetrate the plant cell when they are used in the form of lipophilic organic derivatives, may account for their particular effectiveness as poisons to *Plumaria*. Nevertheless, a question remaining unanswered is that of whether heavy metals are also able to penetrate plant cells when the poisons are used as inorganic salts of low lipoid solubility. So far direct evidence of the penetration of heavy metals in this form has only been obtained in experiments with crustaceans.[3, 15] However, it is hoped that future studies will shed light on this important aspect of the general problem.

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ON DIFFERENCES BETWEEN SOME EFFECTS OF SALICYLATE AND 2 : 4-DINITROPHENOL

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Abstract—The human RBC treated *in vitro* for 2 hr with 3×10^{-3} M salicylate or 5×10^{-5} M 2:4-dinitrophenol, lose in average 11·22 (salicylate), 13·44 (DNP) mEq./l. cell potassium and take up 2·01 (salicylate), 2·89 (DNP) mEq./l. sodium and 40·8 (salicylate), 34·6 (DNP) ml/l. cell water. 2-Nitrophenol, 3-nitrophenol, and 2:4:6-trinitrophenol have no similar effects.

In the RBC of normal persons in whom during acute experiment the serum salicylate level rose to 30–48 mg% similar changes occurred. On the contrary, DNP given (a) to humans in doses which induce a metabolic increase similar to that produced by salicylate, and (b) in higher doses (30 mg/kg) to rats, did not influence the electrolyte and water content of RBC. High salicylate doses induce a water, potassium and sodium diuresis in the rat, but DNP exerts no similar action. DNP shows no considerable effect in rat's hind-paw oedema-test, whilst salicylate inhibits the oedema.

These differences may be explained by their divergent energetic effects in the organism. Although both substances have striking influence on metabolism and uncouple oxidative phosphorylation, DNP increases oxygen consumption parallel with the dose, whilst this effect of salicylate is limited. Therefore, unlike DNP, salicylate seems to induce a considerable energy deficiency state of the cells.

THERE is much similarity in the metabolic action of salicylate and 2 : 4-dinitrophenol (DNP). The uncoupling of oxidative phosphorylation is a basic property shared by them. Both increase the metabolic rate, also both enhance the oxygen consumption of cells and mitochondria and at the same time reduce the uptake of inorganic phosphate. Recently Smith and Jeffrey[28] tabulated these and other analogies. Present observations, however, suggest that, in their actions on the whole organism, there are also some important differences. In this paper we report on the differences in the metabolic rate increasing, the anti-inflammatory, the diuretic and the electrolyte pattern influencing action of these substances.

METHODS

We carried out experiments on human red blood cells (RBC) *in vitro*, as well as on human subjects and on rats.

For the purpose of *in vitro* observations blood was drawn from the antecubital veins of healthy young adults and collected in flasks containing heparin (250 I.U./ml blood). Salicylate was added to make up a concentration of 3×10^{-3} M, DNP of 5×10^{-5} M. The unstoppered flasks were shaken for 2 hr at 37°C in a waterbath. After the first hour glucose supplementation was performed with 1 mg per ml blood. After finishing the procedure, the blood was centrifuged at a rate of 3000 rev/min for 30 min, then plasma and buffy coat were removed, and the RBC haemolysed and the haemolysate

deproteinized with trichloroacetic acid. The potassium and sodium content of the clear filtrate was measured with a Zeiss flame photometer. The water content of RBC was estimated by drying to constant weight. The results were compared with RBC from the same persons treated in the same way, except for adding salicylate or DNP. In preliminary trials, one part of the cells was washed and suspended in Krebs–Ringer phosphate buffer; the other part was incubated in its own plasma. Because the results of both methods agreed well, the use of the latter medium was adopted for the remainder of the study. *In vitro* experiments were also performed by adding 2-nitrophenol, 3-nitrophenol and 2 : 4 : 6-trinitrophenol, respectively, to the RBC producing a concentration of 5×10^{-5} M from them.

Salicylate was administered to healthy young subjects in fasting state in doses of 0·10–0·15 gm/kg *per os*, in the form of sodium salicylate. DNP was given under the same conditions, in doses of 10 mg/kg *per os*, using a 0·5 per cent neutralized solution. Metabolic rate determinations were made and blood drawn from the antecubital veins before and 4 hr after the ingestion of the drug. Electrolyte and water content determinations were carried out using the above-mentioned method. Metabolic rate was measured with Noyons-diaferometer. Serum salicylate level was determined using Loberg's ferrichloride method.

Animal experiments were performed on male rats from an inbred strain. DNP (30 mg/kg) was given intraperitoneally to intact adults, weighing 180–250 g, from a neutral 1 per cent solution. Four hours after injection, the metabolic rate was measured with diaferometer at thermoneutral temperature (29°C). Blood was then collected by cutting the vessels of the neck, and electrolyte as well as water content determinations were made. The same procedures were carried out with control animals. Salicylate was given to the rats, weighing 60–210 g, in doses ranging from 100 to 750 mg/kg subcutaneously. Metabolic rate determinations were made 45–120 min after injection.

The diuretic action of salicylate and DNP was compared in rats. The details of the method are all shown in Fig. 1.

The anti-inflammatory action was tested by the rat's hind-paw oedema test. An extract (0·3–0·8 mg) of bull testicle was dissolved in 0·1 ml saline and injected at the plantar side of the hind-paw. The test was carried out on one paw before and on the other after treatment, and the grade of the acutely induced oedema of the two paws was compared. The inhibition of oedema is described as (a) *good*, if no remarkable oedema develops around the tibia, i.e. the tibial folds remain unchanged, (b) *moderate*, if a slight oedema is present, i.e. the tibial folds tend to disappear, but the circumference of the limb increases only slightly, (c) *no inhibition*, if the circumference of the limb increases remarkably and a pad-like swelling of the dorsal side of the paw can be observed.

It should be mentioned that in preliminary experiments the maximum effects appeared about 4 hr after treatment, and so this point was chosen to compare the effects.

RESULTS

Erythrocytes water and electrolyte pattern

The human RBC incubated with salicylate (mean of fifteen experiments) lost 11·22 mEq./l. cell potassium ($P<0·001$), took up 2·01 mEq./l. cell sodium ($P<0·01$) and 40·8 ml/l. cell water ($P<0·001$). The RBC incubated in the same way with DNP

(mean of fifteen experiments) lost during 2 hr 13·44 mEq./l. cell potassium ($P<0·001$), took up 2·89 mEq./l. cell sodium ($0·01>P>0·005$) and 34·6 ml/l. cell water ($P<0·001$). In RBC incubated with 2-nitrophenol, 3-nitrophenol or 2 : 4 : 6-trinitrophenol no remarkable changes occurred (Table 1).

TABLE 1. *In vitro* EFFECTS OF SALICYLATE, DNP AND SOME OTHER NITROPHENOL COMPOUNDS ON THE ELECTROLYTE AND WATER CONTENT OF HUMAN RBC

	Changes in content of potassium (mEq./l.)	sodium (mEq./l.)	water (ml/l.)
Salicylate	−11·22	+2·01	+40·8
DNP	−13·44	+2·89	+34·6
2-Nitrophenol, 3-Nitrophenol, 2:4:6-Trinitrophenol	+ 0·42	+0·23	−1·0

The administration of salicylate to eight subjects produced serum salicylate levels of 30–48 mg per cent and increases of the metabolic rate of from 34 to 52 per cent, which were accompanied by a diminution in potassium content of RBC of 11·83 mEq./l. ($P<0·001$), an increase in sodium content of 3·95 mEq./l. ($0·01>P>0·005$), in water content with 39·5 ml/l. ($P<0·001$). In previous experiments[16] with rats, the acute water and electrolyte effects of salicylate were found to be the same.

In eight persons receiving DNP, the potassium, sodium and water contents of the RBC showed no significant changes, although the metabolic rate increased from 23 to 43 per cent (Table 2). Even the higher DNP doses given to fourteen rats, failed to have any significant electrolytic effects, but the metabolic rate increased with as much as 119 per cent on average (Table 3).

TABLE 2. EFFECTS OF SALICYLATE AND DNP ON ELECTROLYTE AND WATER CONTENT OF RBC IN NORMAL SUBJECTS

	Increase of O_2 consumption (%)	Changes in RBC of potassium (mEq./l.)	sodium (mEq./l.)	water (ml/l.)
Salicylate	34–52	−11·83	+3·95	+39·5
DNP	23–43	−0·42	−0·5	−3·1

TABLE 3. EFFECTS OF HIGH DNP-DOSES ON THE OXYGEN CONSUMPTION AND ON THE ELECTROLYTE AND WATER CONTENT OF RBC IN NORMAL RATS

O_2 consumption of rats (ml/dm^2 per hr)		Potassium content of RBC (mEq./l.)		Sodium content of RBC (mEq./l.)		Water content of RBC (ml/l.)	
control	DNP-treated	control	DNP-treated	control	DNP-treated	control	DNP-treated
71·6±2·75	157±2·28	84·1±1·11	82·08±2·06	8·8±0·33	8·93±0·29	705·6±5·1	705·1±1·94

Water, potassium and sodium diuresis

As can be seen in Fig. 1 the water diuresis, as well as sodium and potassium excretion increased significantly under the effect of salicylate, whilst DNP had no influence.

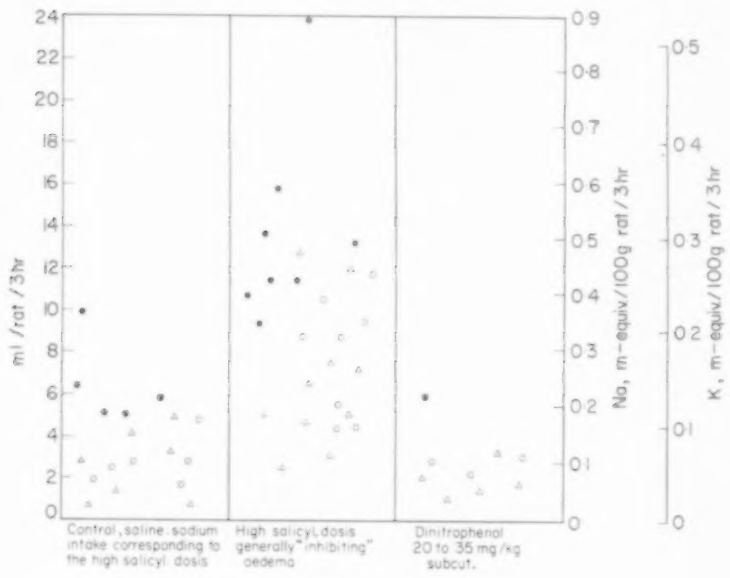
ml / rat / 3hr
Na, m-equiv./100g rat / 3hr
K, m-equiv./100g rat / 3hr
Control, saline sodium intake corresponding to the high salicyl dosis
High salicyl dosis generally "inhibiting" oedema
Dinitrophenol 20 to 35 mg/kg subcut.

FIG. 1. ● sodium; ○ potassium; △ diuresis.

Effects on oxygen consumption

The relation between the doses of salicylate and the oxygen consumption of normal rats is shown in Fig. 2. According to these experiments the increase of metabolism induced by salicylate is limited. We would like to remark that the rate of oxygen consumption was at 4 hr following the injection of salicylate very much the same as after 2 hr, i.e. it did not exceed the limit demonstrated in Fig. 2. On the contrary, it seems that the metabolic increasing effect of DNP runs parallel with the doses and the increase can even exceed 100 per cent (Table 3).

Inhibition of acute experimental inflammatory oedema

The marked anti-inflammatory action of high salicylate doses has been repeatedly demonstrated.[13, 15] The influence of salicylate and DNP on the rat's limb oedema test is shown in Table 4. As can be seen, unlike salicylate, the anti-inflammatory action of DNP is minimal if it possesses such an action at all.

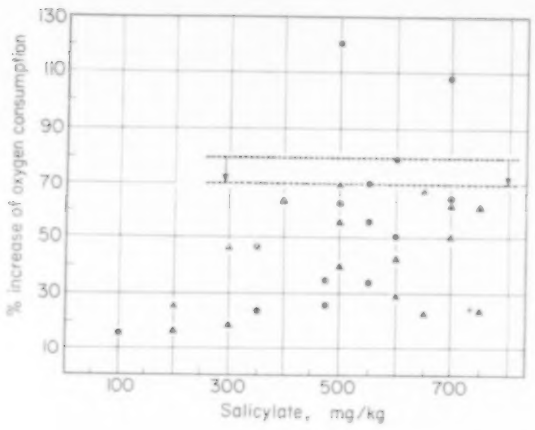

FIG. 2.

TABLE 4. EFFECTS OF SALICYLATE AND DNP IN RAT'S OEDEMA TEST*

	Doses (mg kg)	Number of animals	Distribution of animals regarding the inhibition of oedema 3–4 hr after receiving the drug		
			good	moderate	none
Salicylate	525 to 575 s.c.	32	24	6	2
DNP	5 to 15 s.c.	5	—	—	5
	20 to 35† s.c.	14	5	2	7
	30 i.p.	6	—	1	5

* The oedema test was carried out on the hind-paws of normal rats using testicular extract.
† Although neutralized solutions were injected this was the maximum tolerated dose.

DISCUSSION

A short account of the effects of salicylate exerted on the electrolyte and water content of human RBC has been already published.[32] Our present results show that salicylate and DNP have the same effects on the electrolyte and water content of *in vitro* treated RBC, namely the potassium content diminishes, the sodium and water contents increase. Those nitrophenol compounds (2-nitrophenol, 3-nitrophenol and 2 : 4 : 6-trinitrophenol) which have no metabolic effects, do not influence the electrolyte and water content of RBC. According to our results the action of salicylates as well as of DNP causes a net potassium extrusion from the erythrocytes and not an isomolar exchange of potassium and sodium.

Manchester *et al.*[21] stated that the presence of salicylate *in vitro* leads to a greatly increased loss of potassium from isolated rat diaphragm. In Smith's[29] experiments the incubation of the small intestine of rats with 5 m-moles salicylate caused the swelling of the epithelium. Dianzani and Scuro[8] on adding DNP to liver cell mitochondria, observed a swelling of them. Mudge[22] found that the water content of kidney slices incubated with DNP increases, whilst those nitrophenol compounds

which do not uncouple oxidative phosphorylation have no such effect. According to Harman and Feigelson,[11] DNP causes the swelling of isolated heart-muscle mitochondria. In the experiments of MacFarlane and Spencer,[20] however, swelling of rat liver mitochondria did not occur in the presence of adenosine-phosphate plus DNP, although oxidative phosphorylation was inhibited. The optical method, applied by Price *et al.*[25] showed that with 10^{-4} M DNP plus 1 m-mole adenosine phosphate the swelling of the mitochondria was accelerated.

It is well known that for the maintenance of concentration gradients of potassium and sodium across the cell membrane a steady energy consumption is needed. The chief pathway in production of this energy in the RBC is anaerobic glycolysis. However, the known existence of the very active glucose-6-phosphate and phosphogluconic acid dehydrogenases suggests that they may be part of an aerobic energy yielding system.[24] According to recent observations the hexose-monophosphate shunt really participates in energy production of RBC.[4, 23] It is probable that salicylate and DNP are influencing the energy producing system of the RBC causing an energy deficiency state, the consequences of which are the electrolytic shifts and swelling of the cells.

The results with salicylate and those with DNP respectively, obtained in the *whole organism* (in human beings and in rats) are diverging. The RBC of those persons to whom salicylate was given, underwent similar changes as the RBC treated *in vitro* but DNP *in vivo* exerted no effects on the electrolyte and water content of the RBC. To exclude the possibility that the dose of DNP was insufficient, we performed experiments on rats where 30 mg/kg DNP was given intraperitoneally, i.e. nearly the maximal tolerable dose. This quantity also failed to exert any effects on the electrolytic and water content of RBC. In agreement with this we demonstrated that DNP, unlike salicylate, induces neither water nor potassium or sodium diuresis.

The data of Keitel[12] and Riecker[27] are consistent with the hypothesis that a reduced RBC potassium content is associated with potassium depletion of the average body cell. A parallelism in sodium content is also suggested. We may suppose that there exists some parallelism between our observations made on RBC and those changes which come to pass in the average body cell. In fact our recent experiments with rats seem to agree well with this statement.[17]

To explain the differences in the effects of salicylate and DNP we recall the considerations of Kelemen and Tanos[16] about different energetic consequences of uncoupling of oxidative phosphorylation. They found that if oxygen consumption together with circulatory and other adjustments can compensate for increasing energy demand produced by the uncoupling effect, then signs of energy deficiency are not striking. It is known that salicylate increases oxygen consumption.[5, 6, 7, 14, 30] But it could be observed that in high ranges this increase does not bear a linear relation to the dose. The relation between the doses of salicylate and the oxygen consumption of normal rats shows a limit in the increase of oxygen consumption at about 70 per cent. A similar limit could be observed in the experiments of Tenney and Miller[31] with dogs. The results obtained on humans suggest that although there is a significant correlation between the increase in oxygen consumption and the serum salicylate concentration at the lower and at the therapeutic levels,[2, 3] there exists a similar limit in human beings too, if further increase of salicylate level is produced.[33] Considering these facts an energy deficiency in the organism due to the effect of high doses of salicylate

is probable. Other indirect proofs, namely that the reaction of the organism due to the effect of high salicylate doses is markedly similar to that which occurs during hypoxia[24] support this suggestion. This energy deficiency may be the cause of the electrolyte shifts between the RBC and their environment in the organism.

On the contrary, as already stated DNP increases the oxygen consumption of rats[10] and of human subjects[26] fairly parallel with the dose. Using tolerable doses of DNP, a limit in the increase of oxygen consumption could not be observed. The basal metabolic rate of DNP-intoxicated patients ranges from +100 to +400 per cent.[3a] According to Dianzani and Dianzani-Mor[9] the DNP dose used in our rat experiments (30 mg/kg) reduces the P/O ratio in rat liver cells with 60 per cent. Applying the tentative formula of Kelemen and Tanos[16], an increase of oxygen consumption of 120–140 per cent would be needed to prevent an energy deficit (Fig. 3). As Table 3 shows the oxygen consumption of our rats increased by about this degree, thus a significant energy deficiency does not seem present. We suppose that this explains why there are no disturbances in electrolyte and water balance of the cells.

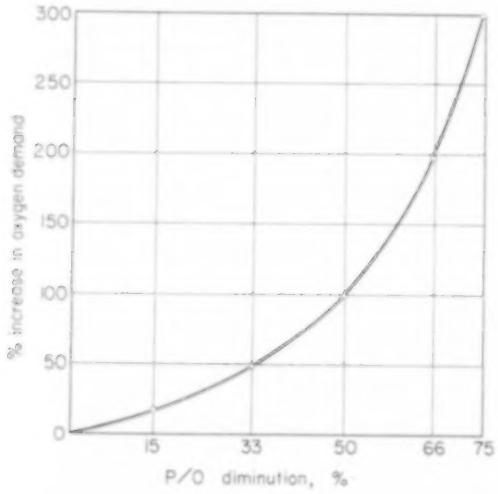

Fig. 3.

Furthermore the anti-inflammatory action of DNP is minimal if not absent, even when the doses approximate to the limit of tolerance. An insufficient adjustment of circulation at high toxic doses of DNP may be the explanation for the few cases in which an inhibition of oedema developed. In agreement with our observations, Adams and Cobb[1] stated that DNP does not diminish the inflammatory reaction in ultra-violet erythema test, which is very distinctly inhibited by salicylate. For these differences in anti-inflammatory action the following explanation may be given. The state of energy deficiency is in some way connected with a hyporeactive, i.e. anti-inflammatory state,[17] therefore salicylate does but DNP does not inhibit the development of oedema in the rat's limb test and in the ultra-violet erythema test.

There are also differences between the action of salicylate and DNP on the carbohydrate metabolism of diabetics.[26] Lehninger *et al.*[18] recently emphasized other differences between certain uncoupling agents, e.g. DNP and thyroxine.

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STUDIES ON GLUTAMIC-OXALOACETIC TRANSAMINASE
THE COENZYMATIC ROLE OF THE *ISO*NICOTINOYLHYDRAZONE OF PYRIDOXAL-5-PHOSPHATE*

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Abstract—The *iso*nicotinoylhydrazone of pyridoxal-5-phosphate has been chemically prepared. It is able to activate glutamic-oxaloacetic transaminase from pig heart, while the cyanohydrin of pyridoxal-5-phosphate is unable to function as a coenzyme. An interpretation of the results has been attempted.

THE present paper deals with the coenzymatic role of the *iso*nicotinoylhydrazone of pyridoxal-5-phosphate. Pig heart glutamic-oxaloacetic transaminase has been chosen as a suitable phosphopyridoxal-requiring enzyme.

Since it was first shown that isoniazid (INH) is capable of inhibiting enzymic activities, heterogeneous results were obtained because of different kinds of materials used, e.g. bacterial cultures, crude extracts of cells, unpurified enzyme preparations (see for references, Hicks and Cymerman-Craig[1]).

In the interpretation of the action of INH on enzyme systems, two alternative hypotheses were given: the competition with pyridoxal-5-phosphate (Py5P)[2] and the interaction with it resulting in the formation of an hydrazonic compound.[3] This latter hypothesis was supported by the chemical preparation of pyridoxal-*iso*nicotinoylhydrazone.[4] Subsequently, the interaction of INH with Py5P was studied by Davison[5] and Wiegand[6] prepared the *iso*nicotinoylhydrazone of Py5P (Py5PINH). Other properties of this compound were investigated by ourselves, and some results on this subject will be briefly referred to here, in connexion with data on glutamic-oxaloacetic apotransaminase.

EXPERIMENTAL

Glutamic-oxaloacetic apotransaminase was prepared from pig heart as described by O'Kane and Gunsalus,[7] and purified up to the fifth step; the resolution was about 95 per cent and the specific activity 3·4 (μmoles of oxaloacetic acid formed by 1 mg protein in 1 min, at 37°C).

The transamination reaction was studied according to the method of Cammarata and Cohen[8] in a Beckman spectrophotometer model DU, equipped with a thermospacer. The temperature variation was of $\pm$ 0·1°C. The amount of L(+)aspartate and α-ketoglutarate was the same in all experiments (20 μmoles in a final volume of 3·2 ml). The resulting molar concentration was higher than the K_m value as determined by Cohen.[9]

* A preliminary report of these results was given at the Fourth International Congress of Biochemistry (Vienna, 1–6 September 1958).

The isoniazid was a gift from Lepetit S.p.A. (Italy); pyridoxal-5-phosphate, L(+)aspartic acid, and α-ketoglutaric acid were obtained from Hoffmann-La Roche S.A. (Switzerland). The *iso*nicotinoylhydrazone of pyridoxal-5-phosphate was prepared in a way similar to the procedure of Sah.[4] The melting point of the compound is >230°C. The elementary analysis has given the following data: (Found: C, 44·87; H, 4·23; N, 15·05; P, 7·95. Calcd. for $C_{14}H_{15}O_6N_4P$; C, 46; H, 4·1; N, 15·3; P, 8·45.) In 0·05 M sodium phosphate buffer, pH 7·4, the orange-red product shows a characteristic absorption spectrum with two maxima at 292 and at 340 mμ. As shown in Fig. 1, the absorption spectrum of the hydrazone is similar to that observed by

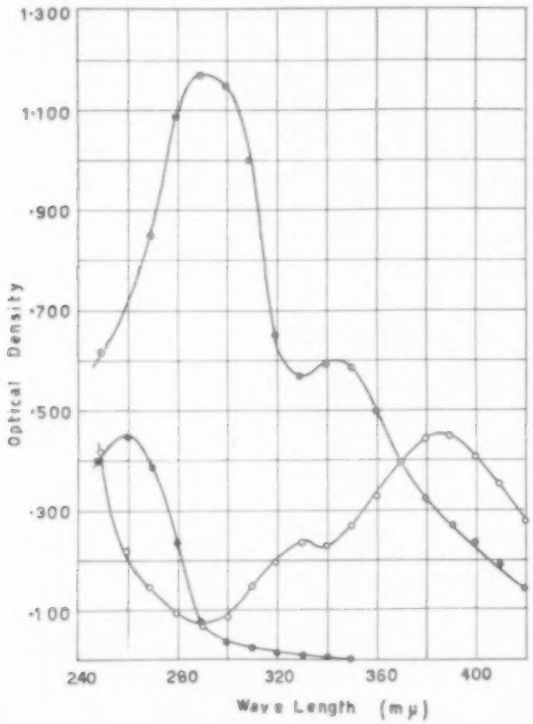

FIG. 1. Absorption spectra of Py5PINH (○), Py5P (○), INH (●), 10^{-4} M, in 0·05 M sodium phosphate buffer, pH 7·4.

Wiegand.[6] Further details on the kinetics of the reaction between INH and Py5P as well as on the pK'_a values and spectrofluorometric properties of the resulting compound will be published in a following paper (see Discussion).

RESULTS

It was noted earlier that INH is capable of inhibiting the activity of glutamic-pyruvic transaminase.[1] To investigate this point with glutamic-oxaloacetic transaminase, INH in increasing amounts has been added to the recomposed enzyme (following incubation with Py5P); there was no inhibition up to 10^{-3} M concentration of the drug. No inhibition took place when INH was added before or simultaneously

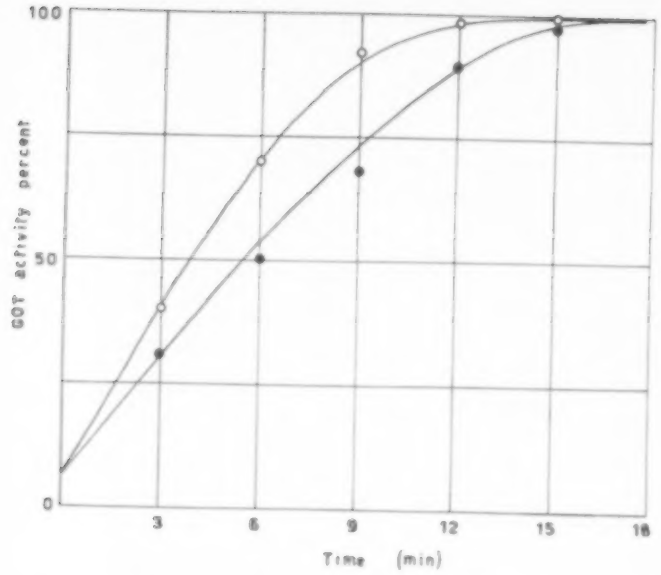

FIG. 2. Glutamic-oxaloacetic transaminase activity as a function of incubation time of the apoenzyme with optimal amounts of Py5P (○) or corresponding amounts of Py5PINH (●). L(+)Aspartic acid was incubated with the enzyme 1 min before starting the reaction with α-ketoglutaric acid. Experimental conditions: 1 mg of protein, 20 μmoles of substrate, and 40 μmoles of Py5P or Py5PINH in a final volume of 3·2 ml. Buffer: 0·05 M sodium phosphate, 7·4. pH

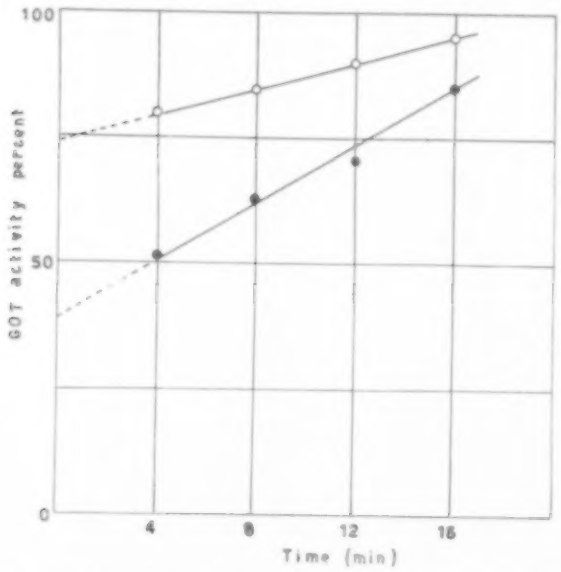

FIG. 3. Glutamic-oxaloacetic transaminase activity as a function of incubation time of L(+)aspartic acid with apoenzyme preincubated with Py5P (○) and Py5PINH (●). Experimental conditions as in Fig. 2.

with Py5P. A slight inhibition (8 per cent) occurred with INH at $3{\cdot}3 \times 10^{-3}$ M concentration, even if isoniazid was added to the recomposed enzyme. It should be noted that the above mentioned concentrations of INH are those existing during the incubation with the enzyme system (30 min). The activity of this latter was measured after 1 : 3 dilution, as required by the spectrophotometric method.

Py5PINH is not a depressant of transaminase activity. On the contrary, it is capable of activating apotransaminase. As shown in Fig. 2, the preincubation time required for maximal activation is longer than that required for maximal activation with Py5P. Figure 3 shows the results of another experiment in which L(+)aspartic acid was added to apoenzyme previously activated with either the native coenzyme or its *iso*nicotinoylhydrazone. The same final activity was observed when the substrate was incubated with the enzyme recomposed with Py5P or Py5PINH.

The optical density at 292 mμ of the solution containing the apoenzyme and the hydrazone is constant during the incubation time and a long time after. No variation was observed even when the substrate, L(+)aspartic acid, was added to the mixture. If the Py5PINH molecule had split, the optical density at 292 mμ would have become very slight (Fig. 1).

DISCUSSION

As suggested by Vilter *et al.*[3] and by Davison,[5] it is possible to explain the INH inhibition of vitamin B_6-requiring enzymes as due to the formation of an hydrazonic compound.

If this is so, Py5PINH should not display properties of coenzyme and should behave like the cyanohydrin of Py5P, which was shown by us in a previous paper to be incapable of acting as a coenzyme.[10] We were therefore astonished when we found that the incubation of the apoenzyme with Py5PINH resulted in the formation of an active enzyme, although longer incubation times were needed than with Py5P.

One could think of two possibilities. Either Py5PINH sets free enough Py5P for interaction with the apoenzyme, or Py5PINH binds itself as such to the apoenzyme. The former instance does not seem very probable (a) in view of the stability of hydrazones, as checked spectrophotmetrically, and (b) in view of the fact that no experimental evidence is available of the enzymic splitting of Py5PINH, although Youatt[11] has shown very recently that there is a small but definite release of pyridoxal from the *iso*nicotinoylhydrazone of pyridoxal in the presence of *Mycobacterium tuberculosis*.

The second possibility is that Py5PINH is bound as such by the apoenzyme, with a subsequent displacement reaction of the following kind:

$$\text{enzyme.Py5PINH} + \text{aspartate} \rightarrow \text{enzyme.Py5P.aspartate} + \text{INH}$$

By such a mechanism, the reaction could start also with the anomalous recomposed enzyme. Of course, after the attachment of Py5PINH to the apoprotein, a physiological holoenzyme should appear. This is perhaps the reason why, as evident from Fig. 3, the inhibition is well defined in the beginning, and disappears with time. Further work will be performed to make this point clear. However, it must be noted that the present results do not permit us to rule out the binding to the apotransaminase by the aldehyde group of Py5P. Conversely the fact that the aldehyde group is bound by the enzyme protein does not rule out its reactivity with amino acids (see also Jenkins and Sizer[12]).

In order to elucidate the discrepancy between the behaviour of the *iso*nicotinoylhydrazone and the cyanohydrin of Py5P, we have made a systematic study of different properties of both the compounds (pK'_a values, fluorescence spectra, etc.). The pK'_a values and the fluorescence spectra have shown a peculiar similarity between Py5P and Py5PINH, while in the cyanohydrin the pK'_a values of phenolic hydroxyl group and pyridine nitrogen are distinctly higher. Thus fluorescence and spectral changes with pH confirm a similarity between Py5P and Py5PINH but a difference between these compounds and the cyanohydrin.[13, 14]

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Note added in proof

While this work was in progress, Gonnard communicated that the *iso*nicotinoylhydrazone of pyridoxal-5-phosphate is able to act as a coenzyme in the decarboxylation of L-dihydroxyphenylalanine (*C.R. Acad. Sci., Paris* **246**, 3539 (1958)).

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REVERSAL OF EFFECTS ON THE RAT NERVE-DIAPHRAGM PREPARATION PRODUCED BY METHYLFLUOROPHOSPHORYLCHOLINES

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(*Received* 1 *November* 1958)

Abstract—The effects of four organophosphorylcholines (methylfluorophosphorylcholine, methylfluorophosphoryl-β-methylcholine, methylfluorophosphorylhomocholine andm ethylfluorophosphorylcarbocholine (3:3-dimethylbutoxyphosphoryl fluoride)) and methyl*iso*propoxyphosphoryl fluoride (Sarin) on the rat nerve–diaphragm preparation have been studied together with the reversal of these effects by various reactivators (2-PAM, MINA, DINA). Sarin and the carbocholine analogue produced neuromuscular block more rapidly than did the quaternary nitrogen compounds. Neuromuscular block produced by Sarin was the easiest to reverse; the reactivators had less effect on the block produced by the organophosphorylcholines particularly in the case of the homocholine analogue.

THE pharmocological properties of the following organophosphorus cholinesterase inhibitors have been described recently:[1, 2] methylfluorophosphorylcholine (I), methylfluorophosphoryl-β-methylcholine (II), methylfluorophosphorylhomocholine

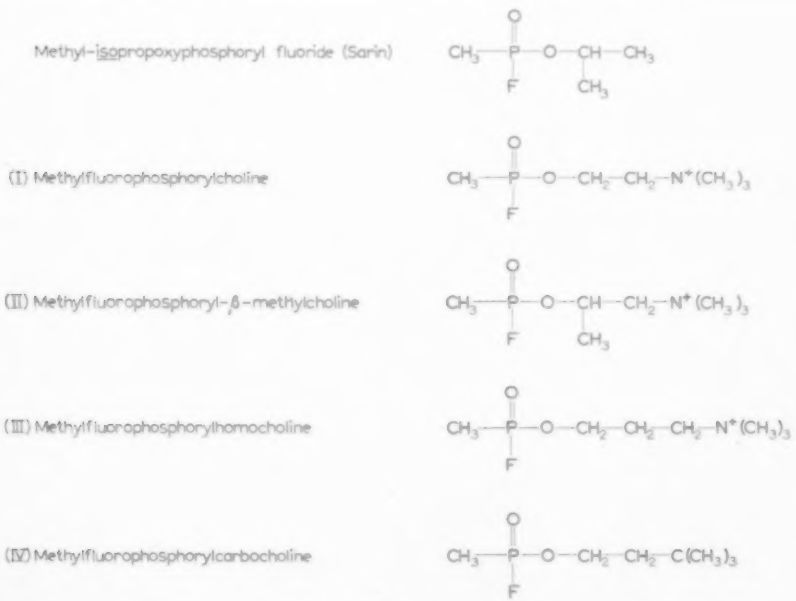

FIG. 1.

(III) and methylfluorophosphorylcarbocholine (IV) (Fig. 1). The quaternary nitrogen compounds are highly active enzyme inhibitors and more toxic than methyl*iso*-propoxyphosphoryl fluoride (Sarin), while the carbocholine analogue is less active as an esterase inhibitor and comparable with Sarin in toxicity. Tammelin[3, 4] reported on the synthesis and cholinesterase inhibition of these and related compounds. The quaternary nitrogen atom of compounds I, II and III is assumed to be attracted to the anionic site of the enzyme. The fourth compound (IV), having no quaternary nitrogen atom, can only react with the esteratic site, which may explain its relatively low affinity for the enzyme. Thus, in the phosphorylated enzyme, the anionic site may be occupied in the case of the quaternary compounds, but not when Sarin and the carbocholine analogue are the inhibitors. This may explain the observation in experiments *in vitro*,[3, 5] with erythrocytes as the source of enzyme, that cholinesterase inhibited by Sarin can be reactivated by pyridine-2-aldoxime methiodide (2-PAM) and analogues, whereas that inhibited by methylfluorophosphorylcholine cannot. The effects of different reactivators on the rat nerve–diaphragm preparation, treated with the four organophosphorylcholines and Sarin, have now been investigated, and the results are reported in this paper.

METHODS

Forty-five adult rats of both sexes, weighing between 170 and 200 g, were used. The isolated phrenic nerve–diaphragm preparation was set up as described by Bülbring[6]. The volume of the bath was 100 ml, containing Tyrode's solution with 2 g of dextrose per 1000 ml. The bath was aerated with a mixture of 95 per cent O_2 and 5 per cent CO_2 with the aid of a sintered glass tube, fixed at the bottom of the bath. The temperature was kept constant at 37°C.

The preparation was taken from the left side of the diaphragm. It was stimulated supramaximally through the phrenic nerve by means of a Grass stimulator delivering rectangular pulses at frequencies of 20 and 40 c/s, and voltages of 0·3 to 0·8 V. The tetanic stimulations at the two frequencies were given alternately with an intervening test period, and the routine procedure was as follows. The preparation was mounted in position and left for 30 min, while the nerve was stimulated with a frequency of 0·5 c/s to obtain an initial stabilization. The frequency was then changed to 20 c/s for 3 sec followed by 27 sec of rest, changed to 40 c/s for 3 sec, followed by 27 sec of rest, again changed to 20 c/s and so forth (Fig. 2). Within 15 min, counting from the time when the stimulation with the higher frequencies began, the response was uniform and the preparation was considered to be stable. A dose of the inhibitor was then added to the bath with the result that the height of the tetanic contractions was reduced. When the inhibitor had been in the bath for 13 min, the reactivator was added without washing. The height of the tetanic contractions increased, usually to the original level. The effectiveness of the reactivator was measured by determining the time required for the contractions to return to 75 per cent of their pre-inhibition value (75 per cent reversal time).

The inhibitors used were the four organophosphorylcholines (Fig. 1, I–IV) and Sarin. All salts were iodides. The reactivators used were pyridine-2-aldoxime methiodide (2-PAM), mono*iso*nitrosoacetone (MINA) and di*iso*nitrosoacetone (DINA). The concentration of the inhibitors in the bath was $4{\cdot}0 \times 10^{-7}$ M, as it was found that this concentration gave a suitable reduction of the twitch, and that of the reactivators

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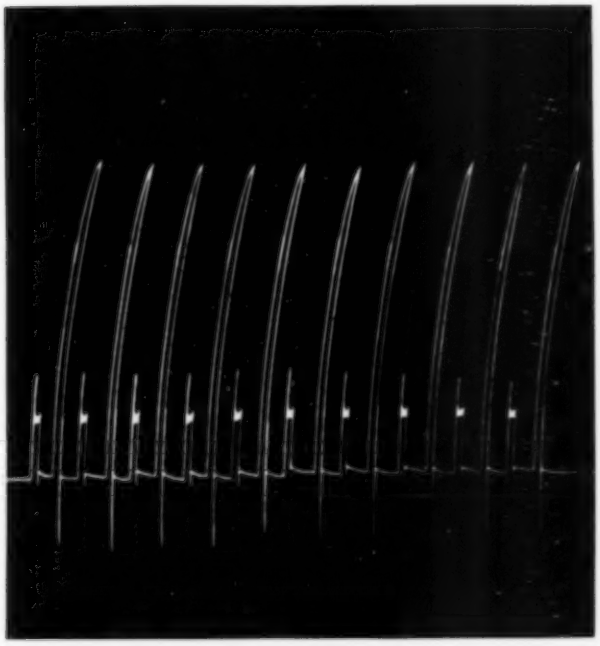

FIG. 2. Constancy of response. Tetanic stimulation of rat nerve-diaphragm preparation at 20 and 40 c/s for 3 sec with 27 sec rest intervals. Drum stopped for 20 sec between each stimulation.

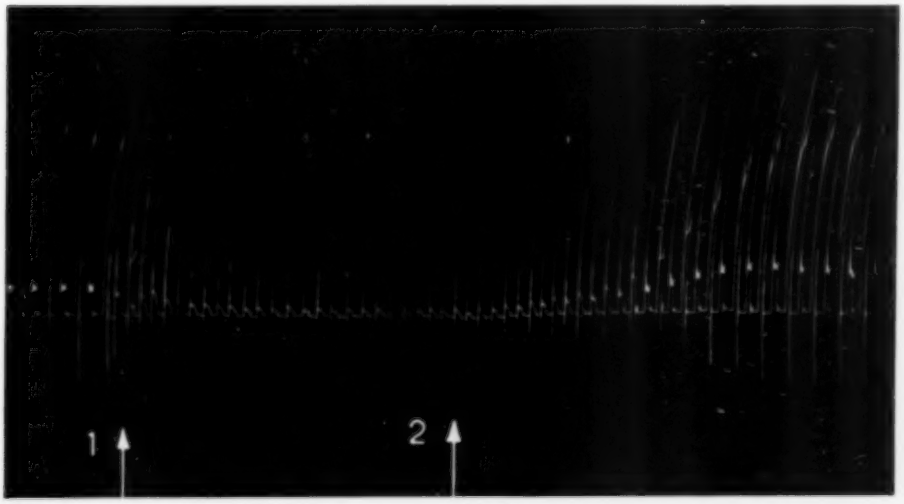

FIG. 3. Reversal of Sarin effects after addition of DINA. Tetanic stimulation of rat nerve-diaphragm preparation with 20 and 40 c/s for 3 sec with 27 sec rest intervals. Drum stopped for 20 sec between each stimulation. At 1: Sarin $4{\cdot}0 \times 10^{-7}$ M. At 2: DINA $1{\cdot}0 \times 10^{-5}$ M.

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$1{\cdot}0 \times 10^{-3}$ M. This reactivator concentration was chosen, as 2-PAM has a blocking effect in higher concentrations.

RESULTS

Constancy of response

When the preparation was in equilibrium the response to the different tetanic stimulations was extremely constant for several hours without addition of inhibitor or reactivator. Variations in the height of the twitch could be observed following washing but the response became constant again within 10 min.

2-PAM of concentrations higher than 3×10^{-3} M had a neuromuscular blocking action, which was reversible after washing (previously also observed by Holmes and Robins[7]). This effect was, however, not noticeable with the lower molar concentration used in the present experiments. DINA and MINA had no visible effects of their own on the twitch in a concentration of 3×10^{-3} M.

Response after addition of inhibitor

There was a rapid decrease of the tetanic response after addition of the inhibitor, even though the immediate response to the tetanic stimulation was increased initially (Fig. 3). Within 13 min the neuromuscular block had reached its maximum. The height of the twitch was then usually less than 20 per cent of the initial one (Fig. 3). Controls showed that there was no spontaneous reversal (tested for 2 hr) when the preparation was not washed. With washing, on the other hand, there was a slight reversal of the response; for this reason the reactivators were added without previous washing.

Maximal neuromuscular block was reached after various periods of time, depending upon the compounds used (Table 1). Sarin and the carbocholine compound were the most rapidly active, the quaternary nitrogen compounds less so.

TABLE 1. TIME REQUIRED TO ESTABLISH MAXIMAL BLOCK OF THE TETANIC TWITCH IN THE RAT NERVE-DIAPHRAGM PREPARATION BY METHYLFLUOROPHOSPHORYLCHOLINES AND SARIN

Inhibitor	Frequency (c/s) 20	40
	Time (min) until maximal block*	
I (-choline)	10	11
II (-β-methylcholine)	12	11
III (-homocholine)	9	10
IV (-carbocholine)	6	6
Sarin	5	5

* Time values are the mean of at least two independent experiments.

Response after addition of reactivator

After the addition of the reactivators a relatively rapid reversal of the inhibitor effects, in most cases to the initial level, was observed (Fig. 3). The 75 per cent reversal time differed for various inhibitors and reactivators as shown in Table 2.

TABLE 2. REVERSAL BY 2-PAM, MINA AND DINA OF THE NEUROMUSCULAR BLOCK PRODUCED IN THE RAT NERVE-DIAPHRAGM PREPARATION BY A SERIES OF ORGANOPHOSPHORUS INHIBITORS

(The reversal is measured by the 75 per cent reactivation time as defined in the text.)

Inhibitor	75 per cent reversal time (min)					
	2-PAM		MINA		DINA	
	Frequency (c/s)					
	20	40	20	40	20	40
I (-choline)	7	10	5	5	9	11
II (-β-methylcholine)	10	11	10	12	10	12
III (-homocholine)	17	16	16	20	12	19
IV (-carbocholine)	10	13	20	20	15	14
Sarin	4	6	5	6	8	10

In a few experiments where the tetanic responses were of the same height before inhibition and after reversal, the procedure was again repeated with the same inhibitor and reactivator. The neuromuscular block appeared more quickly than before, but the recovery was about the same.

DISCUSSION

Regarding the differences in the periods of time until maximal block was produced, the following interpretation of the results seems to be reasonable. Sarin and the carbocholine analogues, not being quaternary nitrogen compounds, easily penetrate into the muscle and rapidly produce neuromuscular block. The slight difference between their effects may possibly be due to the higher molecular weight of the carbocholine analogue, resulting in slower diffusion. Possibly, solubility properties may also be of importance. The slower action of the three quaternary nitrogen compounds might be explained by penetration difficulties, owing to their ionized structure It also seems reasonable that the homocholine analogue, being the most active inhibitor,[3] has the most rapid effect of these three compounds. Studies at different inhibitor concentrations might reveal which of the above mentioned factors is of dominating importance, i.e. diffusion-rate or anticholinesterase activity.

The fact that the addition of 2-PAM results in more rapid reversal of the block produced by Sarin, as compared with the quaternary nitrogen compounds, supports the original theory of Wilson,[8] according to which the potency of this reactivator is

partly due to its attraction to the non-occupied anionic site. The comparatively slow reversal of the neuromuscular block produced by carbocholine might be explained by steric hindrance in the enzyme-inhibition process, as also this inhibitor can be polarized to give an incipient positive charge which may be weakly attracted to the anionic site. In the case of the quaternary nitrogen compounds the rate of reversal seems to be inversely proportional to the enzyme-inhibiting effect, but for the rest it is difficult to explain the differences in reversal of the neuromuscular block.

The mechanism of action of the oximes in reversing neuromuscular block has been explained by reactivation of inhibited cholinesterase.[7] However, it has not been possible to obtain any measurable reactivation of erythrocyte cholinesterase inhibited by methylfluorophosphorylcholine in experiments *in vitro*.[5] However, the results reported in the present communication may be due to a reactivation of the inhibited enzyme in the muscle preparation, or there may be some other mechanism involved, possibly concerned with the receptors.

Acknowledgements—Our thanks are due to the director of this institute, Professor G. Ljunggren, for the kind interest he has shown in this work.

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UNE MÉTHODE POUR L'ÉTUDE DES LIPIDES HÉMATIQUES—I

RAPPORTS ENTRE ACIDES GRAS INSATURÉS, ACIDES GRAS TOTAUX ET CHOLESTEROL*

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(*Received* 23 *September* 1958)

Abstract—A new method for testing the level of blood lipids is proposed. This technique involves the use of α-thocopherol in order to prevent oxidation of fatty acids; it results in a better accuracy in comparison with previous methods.

Average values of blood cholesterol, total fatty acids and unsaturated fatty acids in experimental arteriosclerosis of rabbit are reported. The ratio of iodine to total fatty acids expressed in equivalents is called the "unsaturation index". It increases at beginning of experimentally induced arteriosclerosis, possibly because of the withdrawal of unsaturated fatty acids from the tissues.

PENDANT ces dernières années, plusieurs chercheurs se sont occupés des acides gras insaturés, et particuliérement de l'acide linoléique,[1-9] comme agents thérapeutiques de l'artériosclérose. Ces études ont demontré que l'effet de ces substances s'explique par une action hypocholestérolémiante, et que, introduites à temps dans la diète, elles réussissent à éviter le développement de la maladie. Ces recherches ont ouvert un nouvel horizon thérapeutique à la maladie artériosclérotique, et, en outre, elles ont ravivé les études sur les lipides insaturés. Bien que les études sur ce sujet soient assez nombreuses,[10-12] on n'a pas encore clarifié le mode d'action de ces substances et déterminé le rôle qu'elles jouent dans la physiologie et la pathologie. Des études statistiques[13-15] ont démontré, de façon péremptoire, l'importance des acides insaturés dans la pathogénie de l'artériosclérose: leur absence dans l'alimentation détermine une apparition plus fréquente et plus facile de la maladie. En se fondant sur ces faits, quelques chercheurs ont essayé de donner une explication du phénomène,[10, 11, 16] mais la question n'a pas encore été résolue; notamment aussi, parceque les techniques, normalement employées pour l'étude des lipides, sont encore difficiles et insuffisantes. C'est pour ces raisons que nous avons crue opportun d'entreprendre la présente étude, avec l'espoir de donner une contribution, si minime soit-elle, à ces études d'actualité.

Pour cela, nous avons abordé les recherches avec des techniques convenables pour étudier les relations des acides gras insaturés avec toutes les autres fractions lipidiques du sérum dans diverses circonstances biologiques et pathologiques. Dans cette première note, nous parlerons de la méthode utilsée et des relations entre acides gras insaturés, acides gras totaux et cholestérol dans l'artériosclérose expérimentale. Nous exposerons

* Communication au Le Congres International de Biochemie, Wien, September, 1958.

d'abord les résultats obtenus sur une solution standard, et ensuite les données des expériences sur lapins.

La méthode utilisée est resumée dans la Fig. 1.

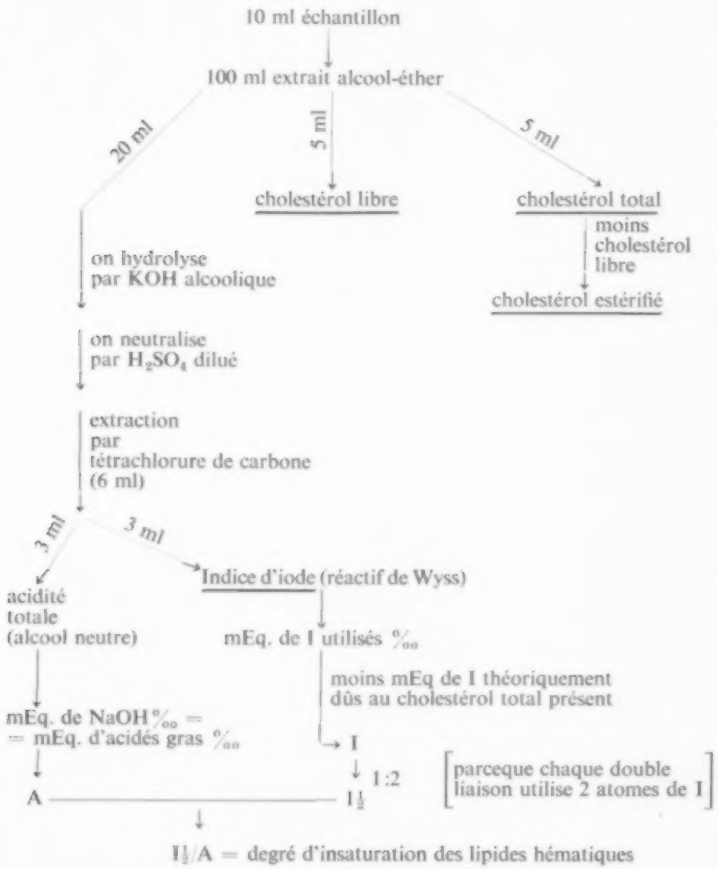

FIG. 1.

De cette manière on réussit à déterminer le degré d'insaturation des lipides et a le confronter avec les fractions cholestéroliques. Le détail le plus important de cette méthode est que le mélange alcool–éther contient 10 mg % de α-tocophérol. Ainsi, on travaille, du dèbut à la fin, en présence d'un antioxydant qui empèche le plus grand inconvénient, de ces méthodes: une notable perte de substance, à cause de la grande facilité avec laquelle les acides gras sont oxydés.

Naturellement, avant d'employer cette substance, nous nous sommes assuré que, par elle même, elle ne changeait pas les résultats d'analyse. Plusieurs déterminations, effectuées avec une solution concentrée de seul α-tochophérol, ont donné des résultats toujours égaux à ceux que fournissait le seul solvant.

TABLEAU I

Sans α-tochophérol						Avec α-tochophérol					
A mEq. d'acides gras (‰)			I mEq. d'iode (‰)			A mEq. d'acides gras (‰)			I mEq. d'iode (‰)		
Théoriques	Trouvés	Perte %	Théoriques	Trouvés	Perte %	Théoriques	Trouvés	Perte %	Théoriques	Trouvés	Perte %
18,5	16,9 ±6	−8,6	14,7	11,1 ±8	−24,5	18,5	17,9 ±1,3	−3,2	14,7	13,6 ±1,75	−7,4

Ces valeurs représentent les moyennes de 100 déterminations. Le cholestérol, déterminé par la methode de Schoenheimer et Sperry, a toujours donné des résultats théoriques.

Dans le Tableau 1 sont resumés les résultats obtenus avec une solution standard contenant: cholestérol 150 mg; acide palmitique 250 mg; acide oléique 250 mg; alcool 100 ml.

L'importance de l'α-tochophérol est bien mise en évidente dans ce tableau. Nous dirons même que se passer de cet antioxydant est pratiquement impossible si l'on veut obtenir des resultats fondés.

En effect, on peut voir que dans le premier groupe de déterminations, sans α-tochophérol, on a une certaine perte de substance: − 8,6% pour l'acidité totale; − 24,5% pour l'indice d'iode. Dans le second groupe, au contraire, ces pertes sont notablement réduites (environs d'un tiers): − 3,2% pour l'acidité totale et − 7,4% pour l'indice d'iode.

En outre, on peut observer que les fluctuations sont beaucoup plus grandes dans le premier cas (± 6 et ± 8) que dans le second, avec α-tochophérol: (± 1,3; ± 1,75).

Considérant ces résultats de cent déterminations avec antioxydant, on peut conclure que la méthode permet de travailler sur du matériel biologique et d'obtenir des résultats assez reproductibles et valables.

Les expériences sur lapins ont étés conduites sur trois lots d'animaux. Le premier était constitué de dix lapins jeunes (trois ou quatre mois) d'un poids moyen de 1 kg; le deuxiéme, de dix lapins âgés (plus de deux ans) d'un poids moyen de 3 kg; le troisième, de cinq lapins (âgés de plus de 1 an) d'un poids moyen de 2 kg, qui ont été examinés avant et aprés traitement au cholestérol selon la technique de Cali et Jacobelli.[21]

Touts les lapins proviennent de notre élevage, où ils sont soumis à des conditions standard, qui ont été maintenues durant nos recherches.

Les résultats, obtenus en travaillant sur des animaux à jeûn depuis douze heures et sur le sang total, sont résumés dans le Tableau 2.

Comme on peut le voir, les différences de valeurs entre les lapins jeunes, vieux et en artériosclerose expérimentale, sont assez notables. En effet, nous voyons que, en allant vers les valeurs élevées de cholestérolémie, 51 mg%, 96 mg%, 147 mg% il y a augmentation parallèle des acides gras dans le sang: 15,2 mEq.‰ 15,8 mEq.‰ 21,9 mEq.‰.

Ces acides gras sont à peu près tous insaturés, car, en comparaison de l'acidité totale, l'indice d'iode augmente en proportion plus grande: de 25 mEq.‰, il passe à 37 mEq.‰ et arrive jusqu à 70,6 mEq.‰.

TABLEAU 2

Nombre et caractures des animaux	I (mEq. ‰)	I½ (mEq. ‰)	A (mEq. ‰)	I½/A	C.T. (mg%)	C.L. (mg%)	C.E. (mg%)	
Lapins jeunes: 10	25	12,5	15,2	0,82	51	34	17	Moyenne de 30 déterminations
Lapins vieux: 10	37,4	18,7	15,8	1,17	96	77,5	18,5	Moyenne de 30 déterminations
Lapins avant le traitement avec cholestérol: 5	36,2	18,1	17,9	1,01	80	60,5	19,5	Moyenne de 15 déterminations
Lapins aprés 20 jours de traitement: 5	70,6	35,3	21,9	1,61	147	117	30	Moyenne de 15 déterminations

I = mEq. de iode‰; I½ = I : 2; A = mEq. de acides gras ‰; C.T. = cholésterol total mg%; C.L. = cholésterol libre mg%; C.E. = cholésterol esterefié mg%.

De même, le degré d'insaturation des lipides hématiques, mesuré par I½/A, augmente sensiblement, passant de 0,82 à 1,17 et jusqu'à 1,61. En conclusion de ces données, on déduit que, en allant vers l'artériosclérose ou vers l'hypercholestérolémie, on a un net accroissement des acides gras insaturés du sang.

Comment interpréter ce phénomène? Au premier abord, il semblerait que cette donnée ne soit pas en accord avec l'hypothèse la plus accréditée chez d'autres chercheurs, à savoir que, parmi de multiples causes, un défaut d'acides gras essentiels intervient aussi dans la pathogénie de l'artériosclérose. Mais ce désaccord, à notre avis, n'existe pas. Considérons toutes les études,[17–19] où l'on a démontré que, chez des animaux recevant une diète hypolipidique, on a une augmentation des lipides totaux et du cholestérol dans le sang. Ce fait a été interprété en supposant que le manque de ces substances dans l'alimentation, provoque leur mobilisation à partir des tissues de dépot avec élévation de leur concentration hématique. Si ceci est exact, on peut penser qu'il en est de même dans notre cas. En effet, si un défaut d'acides gras insaturés (soit-il alimentaire, primaire ou secondaire; on ignore encore son origine) intervient dans la pathogénie de l'artériosclérose, les acides gras insaturés seront mobilisés des tissus et leur taux augmentera dans le sang circulant, au moins pour la premiere phase de la maladie.

Cette interprétation du phénomène acceptée, nous voudrions présenter une autre considération. On a démontré que l'élastine[20] des tissus artériels est essentiellement constituée, en plus d'une protéine et de phosphore, par un lipide insaturé. Si ce lipide insaturé est saturé ou remplacé par un autre lipide saturé, l'élastine se décompose spontanément, perdant toutes ses propriétés. Ce fait peut donner une'explication du mode d'action des acides gras insaturés dans la pathogénie de l'arteriosclérose. En effet, nous pouvons supposer que le tissu, d'ou les acides gras insaturés (quelle que soit la cause de leur defaut) sont mobilisés, soit réellement le tissue élastique. Dans ce cas, l'élastine se décompose, le tissu élastique se désagrége et le procédé artériosclérotique commence à ce moment.

Ces sont là seulement des hypothèses qui devront être confirmées et aux quelles nous travaillons dés à puisent.

Enfin, il nous semble qu les données et les hypothèses présentées s'accordent trés bien avec celles d'autres chercheurs, et qu'elles contribuent, au moins en partie, à clarifier la pathogénie de l'artériosclérose.

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SYMPOSIUM AND CONFERENCE REPORTS

FROM time to time critical summaries will be published of the proceedings of conferences or symposia held on a specific topic pertinent to those interested in the biochemical aspects of pharmacology. Suggestions for conferences which should be reported are most welcome and should be addressed to the regional editors. It is hoped to publish reports of this type within two to three months of the time of the conference.

MONOAMINE OXIDASE INHIBITORS

This is a brief summary, with some personal impressions, of a conference on inhibitors of monoamine oxidase (MAO) held at the New York Academy of Sciences on 20–22 November, 1958.

This conference consisted of reports ranging from structure-activity relationships of new series of MAO inhibitors to the clinical evaluation of these compounds. By far the greatest accumulation of data has come from studies on iproniazid ("Marsilid", Fig. 1) and most of the comments in this report refer to this drug, unless otherwise stated.

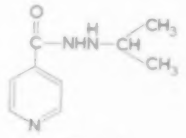

FIG. 1

Studies on the basic biochemistry and pharmacology of these compounds have provided information on relative potencies, onset and duration of inhibition of MAO, and an interesting differential action on various organ systems. β-Phenyl*iso*propylhydrazine (compound JB-516 of the Lakeside Laboratories, Fig. 2) exhibits an *in vitro*-potency against amine oxidase which is about forty times that of iproniazid,

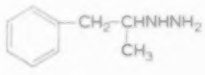

FIG. 2

but this difference apparently becomes much smaller in the whole animal. With iproniazid, the liver enzyme is more rapidly inhibited than the brain enzyme; but with JB-516, the reverse is true.

While it is obviously tempting to attribute most of the actions of these compounds to their inhibition of MAO, and the subsequent accumulation of serotonin and catechol amines, there are certain considerations which seem to have been overlooked by the contributors to the conference in their discussion of mechanism of action. First of all, none of the studies reported was done on a purified preparation of MAO. In fact, it is not even established that MAO is a single, homogeneous enzyme. Secondly, this enzyme is capable of oxidatively deaminating a number of naturally-occurring

amines, which would accumulate when the enzyme is inhibited. Until we learn much more about these amines, it seems unwarranted to attribute all effects of inhibitors of MAO simply to the accumulation of either serotonin or noradrenaline, or both. It is unlikely, for example, that the hypotension, the liver damage, and the hypoglycemia are correlated solely with amine oxidase inhibition.

Another possible mechanism of action of iproniazid, originally suggested by Pletscher and largely ignored at this conference, is the fixation of biogenic amines in their cellular storage sites so that they resist natural or pharmacologic (i.e. reserpine's) releasing mechanisms. Thus, evidence obtained in the laboratory of the writer indicates that iproniazid not only raises the level of 5-hydroxytryptamine in brain through MAO-inhibition, but also inhibits the release of this amine from intracellular granules which ordinarily is elicited by reserpine.

Most of the investigators at this conference justifiably agreed that the clinical properties of these compounds are interesting, promising and worthy of much further work. At present, it would appear that these drugs have some practical value in the following clinical conditions.

1. *Psychiatric disorders*

In general, chronic schizophrenics who are apathetic, depressed and "burned-out" respond much better than those with agitated depression or those classified as acute schizophrenics. In this latter group perhaps as many as 75 per cent may be made worse by these agents. Some clinicians have reported success in refractory patients by using combinations of an inhibitor of MAO and a tranquilizer. One group from Boston (Alexander and Berkeley) illustrated the important point that careful selection of patients for therapy with iproniazid can lead to a much higher percentage of success than poor selection. They found a recovery rate of 88 per cent in a group, classified as psychesthenicanhedonic, characterized by an inability to experience pleasure and satisfaction, a joyless, inhibited state; while there was a recovery rate of only 26 per cent among those exhibiting classical depressive reactions.

The ultimate place of such agents in the therapy of psychiatric disorders, however, must await many more definitive studies. There is a good likelihood that more effective agents in this category will be introduced in the future. This is probably the most important area of their application.

2. *Angina pectoris and coronary disease*

The beneficial action of iproniazid in angina pectoris, first observed by Cesarman in Mexico, was a clear case of serendipity, and today even hints as to the mechanism of this action are few and obscure. Nevertheless, all investigators at this conference were in agreement that excellent results can be expected from iproniazid and presumably, from related compounds. All report relief of pain, and mental changes including euphoria, excitement and loss of perception of the angina. This, of course, may be undesirable because, at least theoretically, it could lead to undue physical activity which might result in sudden coronary insufficiency. There is disagreement about whether iproniazid can correct ECG-abnormalities and change the response to the Master exercise test. Such discrepancy in results may reflect different dosage regimens, which range from 75 mg to 150 mg daily. In view of the long-term therapy required and the toxic hazards of the drug, it is probably desirable to keep the dosage on the

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low side of the above range. One important point which seems to emerge from all the reports is that one obtains no therapeutic results without attendant side-effects.

3. *Diseases of the gastro-intestinal tract*

In a number of conditions like colitis, hepatitis, etc., in which patients suffer from anorexia and malnutrition, one group of investigators reported improvement in appetite and sense of well-being after long-term therapy with iproniazid. Their data strongly suggest that the improvement in appetite is the direct result of a significant reduction in blood glucose. Those patients who had no change in their blood glucose levels reported no subjective improvement. Those patients who reported and showed improvement failed to demonstrate any change in total gastric secretion, free or total hydrochloric acid, liver function, blood sedimentation or blood amylase values. There is little question, then, that improvement in these conditions is the result of the hypoglycemia and the mental changes elicited by iproniazid.

4. *Rheumatoid arthritis*

An interesting paper from the Cleveland Clinic (Scherbel and Harrison) revealed that iproniazid may have some place in the treatment of retarded psychomotor activity, emotional lability and other c.n.s. manifestations occurring in patients with rheumatoid arthritis and related diseases. It was made clear that iproniazid had no effect on the objective signs, nor did it alter the clinical laboratory data. But there was tremendous improvement in the motility and in the subjective symptoms of these patients. Again at this time these very interesting practical results are without explanation on a basic level.

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One important point of controversy arose in the discussion of the clinical use of these agents. This concerned whether, in view of the distinct toxic potential of this group of drugs, they should be relegated to use in hospitals alone, or whether they might be prescribed safely in general office practice. Since most clinicians have agreed that, regardless of the conditions being treated, clinical response to iproniazid is almost always associated with some toxic effects, it seems reasonable at this stage to restrict the drug to use in hospitals, where toxic effects can be detected and dealt with as soon as they appear. The toxicity of iproniazid ranges from annoying side-effects like dizziness, urinary retention and impotence, to serious and sometimes fatal toxic phenomena. The following toxic and side-effects have been described:

1. *Orthostatic or postural hypotension.* Extreme depression of blood pressure is hardly ever encountered. Nevertheless, this can be a most serious reaction in the patient with angina pectoris, because it can, *per se*, precipitate acute coronary insufficiency.

2. *Jaundice and liver toxicity.* The jaundice appears to be hepatocellular in type. Degenerative changes progressing to necrosis of individual liver cells can take place and may have been the cause of the few fatalities attributed to this drug. Liver involvement is most commonly seen when the dosage of iproniazid approaches 150 mg daily.

3. *Hypoglycemia and increased appetite.* This generally leads to an increase in body weight.

4. *Neurological changes* ranging from peripheral neuropathy to psychomotor and behavioral syndromes. Clonus, hyperreflexia, neuralgias, decreased libido, restlessness and insomnia have all been reported.

5. *Miscellaneous.* Nausea and vomiting, constipation, delay in starting micturition have been prominent.

Cesarman made the interesting observation that many of the toxic sequelae resulting from long-term therapy with iproniazid are reminiscent of effects elicited by a ganglionic-blocking agent like hexamethonium. There have been suggestions of an interplay between iproniazid and certain vitamins, notably pyridoxine, and some clinicians, in the hope of reducing toxicity, administer large amounts of pyridoxine together with the drug. It is not possible at this time to evaluate the efficacy of this procedure.

In summary, one may say that the inhibitors of MAO comprise a group of drugs with a considerable range of therapeutic usefulness. They are by no means harmless agents and should therefore be used with due regard for their toxic potential. While their mechanism of action appears deceptively to be related to their potency of inhibition of MAO there is little reason to doubt that other mechanisms are involved. In the opinion of the writer this series of drugs is destined for a considerable degree of clinical use. This should be the more attractive, since none of the present compounds appears to offer any great advantage over iproniazid. Thus, the ideal agent is yet to be discovered.

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SHORT COMMUNICATIONS

Decreased ribonucleotide pyrophosphorylase activity of *Streptococcus faecalis* and L1210 leukaemia resistant to purine antagonists

(*Received* 12 *February* 1959)

KIDDER[1] first postulated that a purine analogue, 8-azaguanine (AZAG), might be converted to a ribonucleotide which could then act as an inhibitor. Subsequent work has strengthened the postulate that ribonucleotide derivatives of 8-azaguanine, or nucleic acids containing the fraudulent nucleotide, are the means by which AZAG exerts its toxic effects.[2]

Elion *et al.*[3, 4] suggested that resistance to 2:6-diaminopurine in *Lactobacillus casei* might arise by loss of capacity to form 2:6-diaminopurine ribonucleotide and Remy[5] obtained direct evidence in support of this postulate. Similarly, it was postulated that 6-mercaptopurine ribonucleotide is an active inhibitor derived from 6-mercaptopurine (6-MP) and that one possible mechanism of resistance to 6-MP might be loss of activity of an enzyme which catalyses the conversion of 6-MP to 6-MP ribonucleotide.[6–9]

Results of microbiological studies with mutants of *Streptococcus faecalis* resistant to growth inhibition by high concentrations of AZAG and 6-MP showed that the resistant organisms failed to metabolize guanine, AZAG, hypoxanthine or 6-MP to the corresponding ribonucleotides in significant amounts; the parent, drug-sensitive *S. faecalis* did form 8-azaguanylic acid and 6-MP ribonucleotide.[9, 10] Similarly, studies *in vivo* demonstrated that AZAG was metabolized to 8-azaguanylic acid and incorporated into nucleic acid in L1210 but not in L1210/8-Aza.[11, 12*] An extension of this work to include cell-free enzyme systems seemed desirable in order to ascertain whether the observed patterns of metabolism of purines and purine analogues in intact cells were a result of altered cell permeability or of alterations in the enzymic composition of the cells. The present report describes the results of a comparison of the capacity of enzymes from drug-sensitive and -resistant *S. faecalis* and L1210 to convert 8-^{14}C-labelled natural purines, AZAG-2-^{14}C and 6-MP-^{35}S to the corresponding ribonucleotides.

Bacterial enzyme preparations were made from washed cells obtained from 500 ml of 18 hr cultures of *S. faecalis* re-suspended in 10 ml of 0·2 M pH 7·6 Tris† buffer. After exposure of the cells to sonic vibration‡ for 5 min at 0°C, the resulting suspension was centrifuged at 25,000 *g* for 1 hr at 4°C. The clear supernatant thus obtained constituted the enzyme preparation. No evidence was obtained to indicate that purine-degrading enzymes were present in the bacterial enzyme preparations. The leukaemic tumours were homogenized and sonically ruptured at 0°C in the same manner as were the bacterial cells (1 g tissue per 5 ml Tris buffer). The supernatant obtained after centrifugation at 25,000 *g* for 2 hr at 4°C served as the soluble enzyme preparation. Degradative activity of such enzyme preparations toward purines was quite low. Conditions for enzymatic reactions were similar to those used by Way and Parks[14] and Lukens and Herrington.[15] After 1 hr incubation of the enzyme preparation (0·5 ml of bacterial enzyme; 0·1 ml of mammalian enzyme), purine or purine analogue (0·2 μc; 0·5 μmole), and 5-phosphoribosyl-1-pyrophosphate§ (1 μmole) in a final volume of 1 ml of 0·1 M

* Lines of L1210 leukaemia used in this study were obtained from Dr. L. W. Law[13]; L1210/8-Aza = 8-azaguanine-resistant.

† Tris = trishydroxymethylaminomethane-hydrochloric acid buffer.

‡ Raytheon 9 kc, 50 W magnetostriction oscillator, Raytheon Corp., Waltham, Mass.

§ The authors are indebted to Dr. J. M. Buchanan and Mr. T. C. French, Department of Biochemistry, Massachusetts Institute of Technology, Cambridge, for an initial gift of the magnesium salt of 5-phosphoribosyl-1-pyrophosphate (PRPP), and to Pabst Laboratories, Milwaukee, for a research sample of this compound.

Tris buffer (pH 7·6), enzyme action was stopped by heat-denaturation. Aliquot portions of the reaction medium were analyzed by one- and two-dimensional chromatography[10, 16] and by paper electrophoresis.[17] Radioactive compounds were detected by exposing chromatograms to X-ray film; percentage conversion of purines to nucleotides was determined by elution of radioactive compounds for counting in internal gas-flow proportional counters.

The results obtained in this study show that the enzymes that catalyse the reactions of guanine, AZAG, hypoxanthine and 6-MP with PRPP to yield the corresponding ribonucleotides are missing, or are inactive, in SF/MP and SF/AZAG (Table 1). The observation that one of the mutants (SF/MP$_{cc}$) retains considerable capacity to convert hypoxanthine and guanine to ribonucleotides, whereas it has lost significantly in capacity to form 6-MP ribonucleotide and 8-azaguanylic acid, suggests that an altered enzyme affinity for 6-MP and AZAG may exist in this particular case. The enzyme preparation from L1210/8-Aza, a neoplasm which is resistant to both AZAG and 6-MP, showed significantly lower capacity to catalyse the reactions of guanine, AZAG, hypoxanthine and 6-MP with PRPP than did a comparable enzyme preparation from the drug-sensitive L1210 (Table 1). Adenine was converted to adenylic acid equally well by enzyme preparations from both sensitive and resistant neoplasms.

TABLE 1. ENZYMICALLY CATALYZED REACTION OF PURINES AND PURINE ANALOGUES WITH 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE: PERCENTAGE CONVERSION TO RIBONUCLEOTIDES*

Enzyme preparation	Substrate					
	Adenine-8-^{14}C	Guanine-8-^{14}C	Hypoxanthine 8-^{14}C	Xanthine-8-^{14}C	8-Azaguanine-2-^{14}C	6-Mercaptopurine-^{35}S
Bacterial cells						
SF/O	99·7	96·0	100·0	100·0	47·3	100·0
SF/AZAG	76·4	1·7	0	100·0	0	0
SF/MP	91·0	1·7	0	100·0	0	0
SF/MP$_{cc}$	89·4	45·4	79·2	100·0	7·4	13·1
Mammalian cells						
L1210	21·3	42·2	32·6	1·1	16·5	23·6
L1210/8-Aza	31·4	11·0	8·7	0·6	2·4	4·3

A sample of 6-mercapto-9-β-D-ribofuranosyl-5′-phosphate synthesized in this laboratory by Dr. H. J. Schaeffer and Miss Jeanette Thomas was observed to migrate with enzymatically synthesized 6-MP ribonucleotide upon co-electrophoresis in formate buffer[17] or chromatography in several solvent systems. The identity of other ribonucleotides was confirmed in a similar manner.

SF/O = parent drug-sensitive *S. faecalis* (ATCC 8043); SF/MP and SF/MP$_{cc}$ = 6-mercaptopurine-resistant mutants isolated and characterized by Dr. Dorris J. Hutchison[15]; SF/AZAG = 8-azaguanine-resistant.[10]

* See text for details.

It is concluded that resistance to 8-azapurines and 6-mercaptopurine in the biological systems studied is a consequence of decreased activity of certain of the ribonucleotide pyrophosphorylases.

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Effects of actinomycin–C upon the pyridoxal phosphate contents of the liver

The fact has been recently observed[1] that the administration of actinomycin-C exerts a considerable inhibiting effect upon the glutamic–aspartic and glutamic–pyruvic transaminasic activities of the liver and spleen tissues. In view of the fact that pyridoxal-5-phosphate acts as coenzyme for both these enzymatic systems, research was carried out to find whether and to what extent the antibiotic can alter the pyridoxal-5-phosphate contents of the liver tissue.

The tests were performed on a lot of twelve rats (average weight: 125 to 150 g) which received a daily dose of 50 μg/kg of actinomycin-C in saline (B group), while another twelve-subject lot (C group) was given an intramuscular injection of 10 mg/kg of pyridoxine hydrochloride immediately after the actinomycin dose. The antibiotic was injected intraperitoneally in all cases. The above treatment was repeated for 7 consecutive days, and the animals were killed by decapitation 12 hr after receiving the last injection, whereupon their livers were aseptically removed, weighed and assayed for pyridoxine and pyridoxal phosphate by the Parrish *et al.*[2] and the Boxer *et al.*[3] methods respectively. The results obtained are summarized in Table 1 together with such data as refer to a further twelve-rat lot (A group) treated for the same period of time with injections of saline.

Table 1. Pyridoxine and pyridoxal phosphate in the liver of rats treated with actinomycin-C (mean values $\pm$ s.e.)

Number of animals	Group	Pyridoxine		Pyridoxal phosphate	
		Conc. (μg/g)	Tot. contents (μg)	Conc. (μg/g)	Tot. contents (μg)
12	A	8·22 $\pm$ 0·54	45·08 $\pm$ 2·13	1·59 $\pm$ 0·34	8·71 $\pm$ 0·65
12	B	6·13 $\pm$ 0·49	34·87 $\pm$ 2·68	0·38 $\pm$ 0·03	2·18 $\pm$ 0·16
12	C	9·36 $\pm$ 0·62	51·66 $\pm$ 3·01	0·52 $\pm$ 0·03	2·87 $\pm$ 0·19

The results clearly show that, while only a slight reduction is determined by actinomycin-C in the pyridoxine contents of the liver (25·5 per cent less than the A group subjects), the antibiotic lowers its pyridoxal phosphate contents to a very substantial extent (86·1 per cent).

Animals of group C, treated with both actinomycin-C and pyridoxine, show no decrease in the pyridoxine contents (which is in fact slightly higher than in the control group), whereas the pyridoxal phosphate level in this group remains far below the normal value (67·3 per cent less than A group animals).

The assumption would therefore seem quite reasonable that the antibiotic mainly affect the conversion of the various free forms of vitamin B_6 into pyridoxal-5-phosphate.

As a matter of fact, a reduction in the coenzymatic functions of vitamins may be mainly determined by any one of the following four conditions: (a) low vitamin availability resulting from either a stepped-up consumption rate or a low-vitamin diet, (b) changes in the vitamin-absorbing capacity of either the stomach or intestine, (c) reduced conversion of free vitamins into the corresponding coenzymes, or (d) inhibition of the coenzymatic activity. Factors (c) and (d) above appear to be the ones most likely to come into play in the case of actinomycin-C, but the fact must be admitted that no particular evidence is offered by our findings that may provide a definite explanation of the chemical or physico-chemical processes through which the antibiotic exerts its action.

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Papers to be published in future issues

Brenda M. Bollard and Henry McIlwain: Cocaine and Procaine on the electrically stimulated metabolism of cerebral tissues.
J. C. Marsh and Mary E. King: Purification of trans-N-glycosidase of *Thermobacter acidophilus:* Inhibition of enzyme by 6-azathymidine.
L. M. Gaudette and Bernard B. Brodie: Relationship between the lipid solubility of drugs and their oxidation by liver microsomes.
E. G. Erdös, F. F. Foldes, N. Baart, E. K. Zsigmond and J. Zwartz: The accelerating effect of narcotic analgesics on the hydrolysis of aromatic substrates by human plasma cholinesterase.
C. E. Carter: Reaction of 6-mercaptopurine with inosine- and guanosine-5'-phosphate pyrophosphorylase purified from E. coli.
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COCAINE AND PROCAINE ON THE ELECTRICALLY STIMULATED METABOLISM OF CEREBRAL TISSUES

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Abstract—The effects of cocaine and procaine on the metabolism of electrically stimulated slices of cerebral tissues have been examined. Cocaine was taken up by the slices to give distribution ratios of 1·2 (grey matter) and 1·4 to 1·6 (white matter) in favour of the tissue, and did not appear to be hydrolysed. Procaine also became associated with the tissue, giving equal distribution between slice and saline, but approximately one-third was hydrolysed to *p*-aminobenzoic acid.

In tissues to which electrical pulses were not applied, 50 μM cocaine caused a small diminution in respiratory and glycolytic rates. The phosphocreatine level was also approximately 10 per cent lower. Maximally stimulated grey matter was more sensitive, 50 μM cocaine causing a 45 per cent reduction in both respiration and glycolysis. Inhibition of response occurred to the same degree in white matter exposed to localized stimulation. The rate of phosphocreatine breakdown caused by electrical stimulation was retarded by approximately 90–97 per cent in the presence of 20 μM cocaine. Likewise the concomitant rate of formation of inorganic phosphate was reduced to a similar extent.

Results obtained using procaine were fundamentally the same as those found for cocaine but it was necessary to use nearly four times the concentrations found effective with cocaine, for the inhibition of stimulated respiration and glycolysis.

LABORATORY investigation of the action of cocaine has mainly concerned the effects on peripheral nerve which are the basis of its application as a local anaesthetic. Its central properties have received less study, although the plants which afford cocaine first appear to have attracted attention as stimulants and it is this property which has led to the adoption of cocaine as a drug of addiction.

Following previous studies of excitant and depressant drugs on electrically stimulated cerebral tissues,[15, 17, 19] cocaine has therefore been examined from this point of view. Also, because its action as a local anaesthetic concerns neural conduction the newly developed techniques[3] for observing the spread of excitation in isolated cerebral tissues have been applied. It was further felt valuable to compare the properties of cocaine with those of procaine, which is a local anaesthetic less potent as a cerebral excitant. Cocaine is active as a central excitant at concentrations much lower than those at which it is used as a local anaesthetic, and therefore in the present experiments the concentration of the drug has been determined in the tissues on which it was acting.

EXPERIMENTAL

Drugs

Cocaine and procaine (British Drug Houses) were prepared as neutral solutions of the hydrochlorides such that 0·1 ml added to 3 ml of experimental saline gave the required concentrations.

For estimation of cocaine, samples of tissue at the end of manometric experiments were removed with a mounted, bent wire, rinsed for 1 sec in cocaine-free saline and ground in 1 ml saline; 2 ml of 1·5 M phosphate, pH 7·0 was added and the drug extracted with 15 ml benzene. The benzene extraction was applied also to 1 ml of the saline in which the tissue had been incubated, and the cocaine in the extracts determined according to Woods *et al.*[25] Tissues which had not been in contact with cocaine gave a small "apparent cocaine" of approximately 6 μmoles/kg of tissue when determined by this method, and this value was determined in each experiment and subtracted from the values found in the presence of the drug.

After experiments with procaine, rinsed slices (see above) were ground in 3 ml of 5 per cent (w/v) trichloroacetic acid and both procaine and the *p*-aminobenzoic acid which it yields on hydrolysis, were determined according to Ting *et al.*[24]

Tissue metabolism

Glucose salines buffered at pH 7·4 with phosphates or with 50 mM glycylglycine were prepared according to Kratzing[14]. Slices of guinea pig cerebral cortex were cut and fitted to the silver-grid electrodes *H*, as described by Ayres and McIlwain[1]. These electrodes are described as giving a *distributed* or *maximal* stimulation. Preparation of slices of subcortical white matter, and the use of electrodes *L* for *localized* stimulation of the tissue, followed the description of Bollard[3]. Electrodes *L* and *H* fitted conical manometric vessels of about 20 ml, containing 3 ml of saline equilibrated with oxygen. Respiration was followed manometrically by conventional methods, taking pressure readings each 5 or 10 min; vessels with the tissue were placed at 37°C, 25–30 min after death of the animal. At the end of experiments, samples of fluid were taken for determination of lactic acid according to Barker and Summerson[2].

Experiments on rapid changes in tissue phosphates employed the quick-transfer electrodes of Heald and McIlwain[11]. Slices of 100–120 mg were incubated in 7 ml of oxygenated glycylglycine saline for 30 min before pulses were applied. Immediately after application of pulses for the different periods indicated in Fig. 3, slices were transferred to 3 ml of 10 per cent (w/v) trichloroacetic acid at 0°C and ground. The procedure described by Heald[10] was followed to obtain the barium salts of phosphates insoluble in water, and those soluble in water but insoluble in ethanol, and to determine inorganic phosphate in the former and creatine phosphate in the latter.

RESULTS

Fate of the drugs

Cocaine added to saline with cerebral tissues under the conditions of the present experiments, appeared in the slice but underwent no appreciable loss in total quantity (Table 1). Distribution of cocaine was in favour of the tissue: in grey matter by a factor of about 1·2 and in white matter, of from 1·4 to 1·6. Yutakia[27] noted cocaine to be hydrolysed by certain animal tissues but not by the brain.

Procaine under similar circumstances was partly hydrolysed (Table 1) so that *p*-aminobenzoic acid was found in cerebral tissues at about one-third of the level of the procaine; the procaine itself was distributed in favour of the tissue so that the final tissue level was about the level added to the saline. Ground cerebral tissues also caused breakdown of procaine, as noted by Kisch *et al.*[13] in other tissues.

TABLE 1. DISTRIBUTION OF DRUGS BETWEEN TISSUE AND FLUID

Drug; time of incubation (min)	Tissue	Concentrations (μmoles/l. or kg)			
		In fluid		In slice	
		Drug	Product	Drug	Product
Cocaine; 50,100	None	51	—	—	—
50	Cerebral cortex	52	—	59	—
100	Cerebral cortex	48	—	60	—
50	Subcortical white	52	—	70	—
100	Subcortical white	52	—	82	—
Procaine; 100	None	185±7	—	—	—
100	Cerebral cortex	149±6	4±1	222±11	75±6
100	Ground cerebral cortex	160±7	9±1	—	—

Slices of about 50 mg were incubated in 3·5 ml of glucose-phosphate salines with and without added substances: cocaine initially at 50 μM and procaine at 200 μM. Results are the mean of duplicate determinations except when followed by the s.e., when they represent from nine to fourteen values.

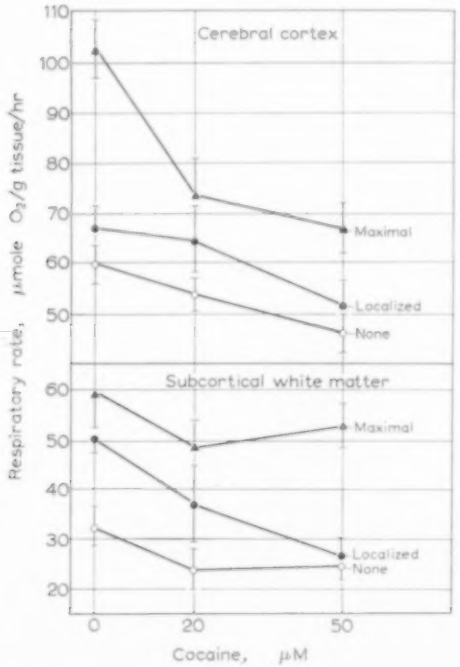

FIG. 1. Cocaine and respiration of cerebral tissues. Slices of cortical grey and of subcortical white matter were held in grid electrodes of types appropriate to give the stimulation indicated. Maximal stimulation (electrode *H*), ▲. Localized stimulation (electrode *L*), ●. No pulses applied, ○. The slices were incubated in glucose-phoshate media without or with cocaine initially at the concentrations quoted. When pulses were applied they followed a period of 50 min incubation without pulses, and were applied for 50 min. Points give the mean respiratory rates of from four to seven determinations and vertical lines indicate the s.d. of the means. Pulses of time constant 0·4 msec, peak potential 10 V, were applied at 100/sec.

Metabolic effects of the drugs

Cocaine. In absence of stimulation, cocaine at concentrations up to 50 μM caused small but significant dimunution in respiration, glycolysis, and phosphocreatine level: cocaine at 20 μM decreased the normal respiratory rate of cerebral cortex by some 4 μmoles/g per hr. Much greater effects were seen in tissues receiving pulses: when maximally stimulated the diminution caused by cocaine was 26 μmoles/g per hr, so that pulses which ordinarily brought about an 80 per cent increase in respiratory rate, caused an increase of 34 per cent (Fig. 1). When subcortical white matter was maximally stimulated, though the proportional increase in respiration was about the same as in grey matter, cocaine at 20 or 50 μM had little effect, the small diminution caused by the drug being similar to that which it caused in absence of pulses. Locally stimulated white matter was however more susceptible: pulses normally increasing respiratory rate by 50 per cent, increased it by 37 per cent in the presence of 20 μM cocaine and only 8 per cent with 50 μM cocaine.

Glycolytic response by each tissue to each type of stimulation was susceptible to 20 or 50 μM cocaine (Fig. 2), in much the same fashion as their respiratory response.

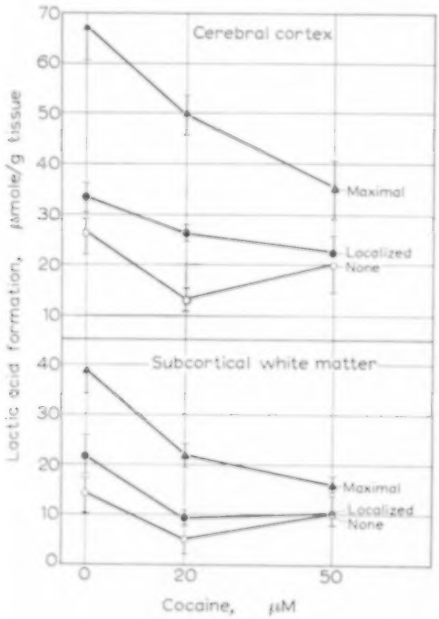

FIG. 2. Cocaine and the formation of lactic acid by cerebral tissues. Lactic acid was determined in 1 ml samples of fluid media taken at the end of the respiratory measurement quoted in Fig. 1. Expression of results: as in Fig. 1.

Cocaine exerted a large effect on the inorganic and creatine phosphates of maximally stimulated cerebral cortex (phosphates were not examined in white matter or following local stimulation). The rate of breakdown of phosphocreatine in absence of the drug, has been observed to be about 1200–1400 μmoles/g per hr.[9, 10] In the presence of 20 μM cocaine, such breakdown was greatly retarded, occurring in the experiments of Fig. 3 at 30 μmoles/g per hr or less. Fig. 3 does not exhibit the maximal rate of

breakdown of phosphocreatine in the absence of the drug, as sufficiently brief periods were not examined; but the values at 10 sec indicate a minimal rate of breakdown of 300 μmoles/g per hr. Thus 20 μM cocaine is inhibiting the rate of breakdown by 90–97 per cent. It also greatly retards the appearance of inorganic phosphate.

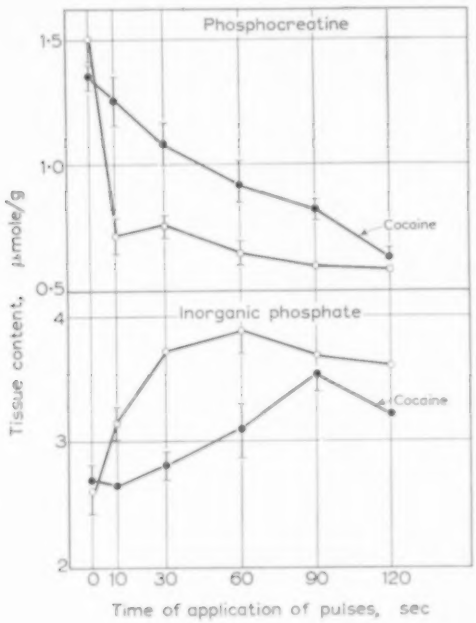

FIG. 3. Changes in tissue phosphates with the application of electrical pulses: ●, with 20 μM cocaine added; ○, with no cocaine. Slices of cerebral cortex, after preliminary incubation in phosphate-free glucose saline (see Experimental) were exposed to condenser pulses of 0·4 msec time constant, peak potential 15 V, at 100/sec for the periods illustrated. They were then rapidly transferred to 10 per cent trichloroacetic acid, extracted, and phosphates determined. Points represent the mean values from three to eight determinations and the vertical lines, the s.d. of the means.

Procaine. Cerebral respiration and glycolysis proved less sensitive to procaine than to cocaine. In the absence of pulses, little or no change in respiration occurred at concentrations up to 200 μM procaine (Fig. 4). Cerebral cortex was again more sensitive than white matter when stimulated maximally; 90 μM procaine then diminished its respiratory response to less than half the value found in absence of the drug. Effects of procaine on glycolysis followed approximately the same course as that described in Fig. 4 for action on respiration. In absence of pulses, glycolysis by cerebral cortex was unaffected by 90 μM procaine. With subcortical white matter, an initial glycolytic rate of 24 μmoles/g per experiment of 115 min was decreased to 17 μmoles. With pulses, glycolysis by maximally stimulated cerebral cortex, again proved more susceptible than did that of white matter. With cerebral cortex, 90 μM procaine decreased lactic acid formation from 59 to 38 μmoles/g tissue per experiment.

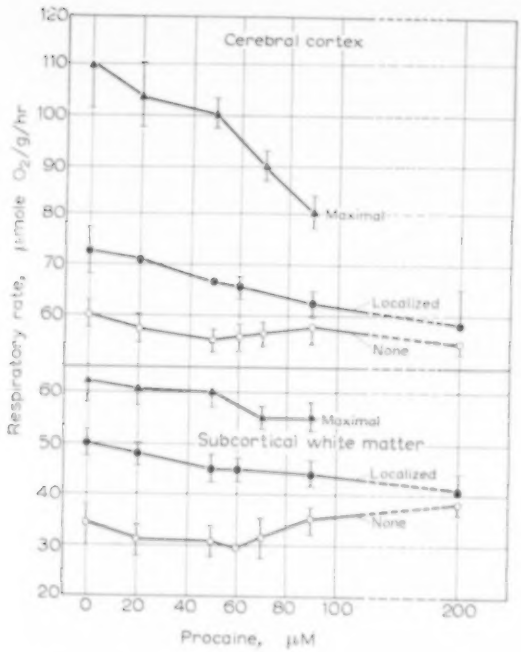

FIG. 4. Procaine and respiration of cerebral tissues. Sliced tissues were examined as described in Fig. 1. The incubating saline containing procaine hydrochloride at the concentrations stated. Stimulation as follows: ▲, maximal; ●, localized; ○, none.

DISCUSSION

Action in vivo *and* in vitro

The electrically stimulated metabolism of cerebral tissues has been found to be extremely sensitive to cocaine and procaine. The concentrations of procaine active in the present experiments are 0·02–0·1 mM, which are to be compared with the 1–2 per cent solutions of procaine used clinically in nerve-blocking; these are 40–80 mM. Such solutions undoubtedly suffer dilution before or during their action. More relevant, therefore, are experiments in which procaine given peridurally was determined chemically in the fluid immediately around the spinal cord and concentration compared with effect; Frumin *et al.*[6] then concluded that partial block was associated with 0·1 mg of procaine/ml, and complete block with 0·2 mg/ml. These are 0·4–0·8 mM, and in the present experiments response in cerebral white matter was largely inhibited by 0·2 mM procaine. This relatively close similarity between the concentrations of procaine required to antagonize the effect of electrical stimulation and those found to occur during its clinical use, contrasts markedly with the high concentrations of 20 mM described by Geddes and Quastel[8] as necessary to inhibit partially the respiration of cerebral tissues stimulated by potassium salts. Many central effects of cocaine, including actions on temperature control and on the electroencephalogram, are observed at from 3–40 mg/kg, or from 0·01–0·14 m-moles/kg.[20, 23] Moreover, Woods *et al.*[26] found the level of cocaine in plasma at the time when it was causing convulsions in dogs, to be 0·01–0·02 m-moles/l. These values are again comparable to the levels

of 0·02–0·05 mM, or 0·02–0·05 m-moles/l., at which cocaine acts in the present experiments.

Grey and white matter

The present results consistently show that grey matter is more susceptible than white to cocaine and procaine, and also that cocaine is the more potent of the two compounds with respect to action on the cerebral tissues studied. The greater susceptibility of grey matter is presumably akin to the finding of Gasser and Erlanger[7] that, in peripheral nerve, the fine, non-myelinated fibres are more susceptible than myelinated fibres. In the present experiments selectivity is exhibited with respect to both respiratory and glycolytic responses and to both cocaine and procaine. That cocaine is the more active substance in the isolated tissue may be related to its greater central effect. It is also notable that cerebral white matter became more susceptible to cocaine when stimulated locally. Accepting the probable conclusion of Bollard[3] that this local stimulation shows a conducted response not seen in the tissue maximally stimulated, the present result suggests processes of conduction to be more susceptible to cocaine than those concerning the resting potential.

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Effects on phosphates

The most impressive effects of the drugs in the present experiments are seen, however, not in the respiratory and glycolytic measurements just discussed, but in the changes in tissue phosphates caused by electrical pulses. With cocaine at only 20 μM, breakdown of phosphocreatine was reduced to about 3 per cent of its normal rate. The final level reached in the presence of cocaine was similar to that in its absence, but was reached in 2 min rather than in a few seconds. Maintenance of phosphocreatine represents a balance between processes of its synthesis and breakdown, the synthesis largely depending on tissue respiration. Cocaine depresses respiration to a small extent in absence of applied pulses, but to a much greater extent in the presence of electrical pulses. This depression is evident in the first few minutes after application of pulses: that is, in the period in which breakdown of phosphocreatine is retarded. Cocaine can therefore be concluded to be opposing the processes normally involved in breakdown of phosphocreatine with pulses.

This stabilization of phosphocreatine can be compared with two other effects of comparably low levels of cocaine. At 20 μM concentration, it has been observed to delay the decline in standing and in action potential caused in frog sciatic nerve by anoxia.[21] Also, cocaine inhibits the loss of K^+ and gain of Na^+ brought about in the squid axon by depolarization.[22] If, as appears likely, the course of action of cocaine in the different biological systems is similar, the initial effect can be seen as a depression of the ion movements associated with activity, so minimizing the utilization of energy-rich substances including phosphocreatine, and the respiration and glycolysis which maintain them. Isolated cerebral tissues as used in the present experiments maintain resting potentials and for this require glucose and oxygen,[16] the consumption of which maintains and is regulated in the tissue in accordance with its content of labile phosphates.[18] The small inhibition of respiration by cocaine in absence of pulses, increased in degree when respiration is increased by pulses, may thus reflect a comparatively small level of ion movement in absence of pulses increased by excitation, as has been observed in sepia axon by Keynes[12].

To judge by effects on tissue phosphates, the processes outlined are much more susceptible to cocaine than to other agents which have been examined. Greengard and McIlwain[9] found the electrically-induced breakdown of phosphocreatine to be unaffected by anticonvulsants, and Cohen and Heald[5] found phenobarbitone to be required at about 1 mM for partial inhibition of the breakdown. This contrasts with the large effect of 0·02 mM cocaine in the present experiments. It is concluded that these experiments exhibit actions of cocaine *in vitro* which give a very real basis for its actions *in vivo*.

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RELATIONSHIP BETWEEN THE LIPID SOLUBILITY OF DRUGS AND THEIR OXIDATION BY LIVER MICROSOMES

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Abstract—The oxidative dealkylation of foreign N-alkylamines by rabbit liver microsomes appears to be limited to compounds which are lipid soluble, as shown by high chloroform to water partition coefficients at physiologic pH. Since the microsomal hydroxylation of aromatic compounds also appears to be limited to lipid soluble substances, it is suggested that an intracellular fat-like boundary separates normally occurring polar substances from the highly non-specific microsomal enzymes.

The dealkylation of foreign alkylamines is catalysed by at least two enzyme systems in liver microsomes.

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In recent years, enzyme systems that catalyse the dealkylation of foreign alkylamines have been the subject of a number of studies. Mueller and Miller[1] reported that 4-dimethylaminoazobenzene and related dyes are demethylated by a rat enzyme system which requires TPN, DPN and oxygen, and that the reaction does not involve transfer of the methyl group, but its oxidation to yield formaldehyde. La Du *et al.*[2] demonstrated that N-dealkylation is accomplished in rabbit liver microsomes by a mechanism that is TPNH-dependent and requires oxygen. The system removes methyl, ethyl and butyl groups. For example, monomethyl-4-aminoantipyrine (MMAP) and its ethyl and butyl analogues are converted to 4-aminoantipyrine, the methyl group appearing as formaldehyde and the ethyl groups as acetaldehyde. The microsomal system dealkylates a variety of N-alkylamines, including methylamphetamine, methadone, methylaniline, ethylaniline, pethidine, mepacrine,[2] diacetylmorphine and codeine.[3]

Normally occurring alkylamines are also dealkylated in the body. Mackenzie *et al.*[4] have demonstrated that sarcosine and dimethylglycine are demethylated by highly specific enzyme systems present in mitochondria. In addition, a number of other methylated amino acids are demethylated by an acetone extract of rabbit liver or kidney.[5]

The present report is concerned with the dealkylation of a diversity of alkylamines by rabbit liver microsomes. Despite its non-specificity, the microsomal system does not demethylate sarcosine and dimethylglycine. The evidence suggests that microsomal

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dealkylation is limited to compounds which are lipid-soluble and that at least two different enzyme systems are involved.

METHODS AND MATERIALS

Materials

β-Diethylaminoethyl diphenylpropylacetate HCl (SKF 525-A) was donated by Smith, Kline and French Laboratories. Triphosphopyridine nucleotide (TPN) was purchased from the Sigma Chemical Company. Reduced triphosphopyridine nucleotide (TPNH) was made chemically[6]. A preparation of glucose dehydrogenase was made from hog liver.[7]

Isolation of tissue microsomes for enzyme studies

Tissue samples were prepared at 0–3°C. Male albino rabbits (New Zealand White) were killed by a blow on the head and the livers were immediately exposed and perfused with 1·15 per cent (isotonic) KCl solution. Livers were removed and homogenized in a Waring blender with 10 vols. of isotonic sucrose, containing 0·2 per cent nicotinamide. The homogenate was strained through cheese cloth and centrifuged at 9000 × *g* for 20 min to remove unbroken cells, nuclei and mitochondria. The supernatant fraction, containing microsomes and soluble fraction of the cell, was carefully decanted and then centrifuged at 78,000 × *g* for 1 hr. Microsomes were separated, washed once with isotonic sucrose solution and recentrifuged. Enzyme activity was assayed in freshly prepared microsomes or in microsomes that had been dried by lyophilization after suspension in water. Microsomes were suspended in 0·1 M phosphate buffer, pH 7·4 in a volume equivalent to that of the original liver. With the lyophilized preparation this corresponded to a quantity of microsomes having a protein content of about 40 mg as measured by the method of Warburg and Christian[8]. Lyophilized micromes were as active as freshly prepared microsomes and were stable for several months.

Analytical methods

4-Aminoantipyrine[2] and aniline[9] were estimated by methods previously described. Formaldehyde and acetaldehyde were trapped by 0·01 M semicarbazide in the incubation mixture and assayed as previously described.[2]

The distribution of various substances between chloroform and water was determined by shaking solutions of each substance in 0·1 M phosphate buffer (pH 7·4) with an equal volume of chloroform* for 20 min. The distribution of each substance is represented as the percentage in the organic phase.

Measurement of dealkylation activity

Previous results have shown that alkylated derivatives of 4-aminoantipyrine and aniline yield amine and aldehyde in equivalent amounts[2]. Accordingly, the dealkylation of these compounds was measured by the amount of primary amine formed. The dealkylation of the other alkylamines was estimated from the amount of aldehyde produced.

One millilitre of the microsomal preparation was incubated with 5 μmoles of substrate, 100 μmoles of nicotinamide, 75 μmoles of $MgCl_2$, 100 μmoles of neutralized

* Chloroform was purified by successive washes with 1 N NaOH, HCl and water.

semicarbazide hydrochloride, 0·2 μmole of TPN, and a TPNH-generating system. The TPNH-generating system consisted of either 30 μmoles of glucose-6-phosphate plus 1·0 ml of a liver supernatant fraction prepared at 78,000 $\times$ g and used as a source of glucose-6-phosphate dehydrogenase, or 40 μmoles of glucose plus 0·4 ml (720 units) of a glucose dehydrogenase preparation[7] and water to make a final volume of 5·0 ml. The reaction mixture was incubated for 1 hr at 37°C in a Dubnoff metabolic shaking incubator in an atmosphere of air. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5 per cent. The proteins were removed by centrifugation and the filtrate was analysed for aldehyde or for primary amine. Enzyme activity was expressed in μmoles of amine or aldehyde formed in 1 hr. With from 1 to 5 μmoles of MMAP as substrate, dealkylation followed a first-order reaction.

EXPERIMENTAL

Dealkylation of various alkylamines by liver microsomes

In Table 1 are tabulated the dealkylation rates of a variety of alkylamines, normal body substrates as well as drugs. The alkylamines are arbitrarily divided into two

TABLE 1. LIPID SOLUBILITY AND DEALKYLATION OF A NUMBER OF ALKYLAMINES BY LIVER MICROSOMES

% Compound extracted into $CHCl_3$	Compound	Relative* activity
>95	Monomethyl-4-aminoantipyrine	100
>95	Ephedrine	110
>95	Pethidine (demerol)	95
>95	β-Diethylaminoethyl diphenylpropyl-acetate	90
>95	Caramiphen (parpanit)	80
>95	1-Phenyl-2-ethoxydiethylamino-3:5-dichlorobenzene	60
>95	N-Methyl aniline	60
>95	N-Ethyl aniline	60
>95	Mepacrine (atabrine)	60
>95	N-Butylaniline	55
>95	Methadone	50
>95	Dimethyl-4-aminoantipyrine (amidopyrine)	34
>95	Levorphan (levo-dromoran)	30
>95	Diphenhydramine (benadryl)	30
>95	Physostigmine	30
>95	Caffeine	20
12	Theophylline	0
11	Theobromine	0
8	Dimethylserotonin	0
<3	Pethidine acid	0
<3	Trimethylphenyl ammonium chloride	0
<3	Adrenaline	0
<3	Sarcosine (monomethylglycine)	0
<3	Dimethylglycine	0
<3	Monomethylaminoethanol	0
<3	Dimethylaminoethanol	0
<3	Choline	0
<3	Creatine	0

Lyophilized microsomes equivalent to 1 g of rabbit liver were incubated with 5 μmoles of substrate as described in "Methods". Glucose and glucose dehydrogenase were used as the TPNH-generating system.

* An activity of 100 is equivalent to the dealkylation of 1 μmole of substrate per hour.

groups, those extracted 95 per cent or more into chloroform, and those extracted 15 per cent or less. It is evident that the foreign compounds which underwent measurable dealkylation were highly lipid-soluble at physiologic pH, as evidenced by their high chloroform to water partition ratios. In contrast, poorly lipid soluble alkylamines, whether drugs or normally occurring substrates, were not dealkylated by microsomes.

Evidence for more than one dealkylation system in microsomes

A comparison was made of the dealkylation rates of various N-alkyl derivatives of 4-aminoantipyrine and aniline. MMAP was dealkylated much more rapidly than the other N-alkyl derivatives of 4-aminoantipyrine. In contrast, monomethylaniline was dealkylated at about the same rate as its dimethyl, monoethyl and monobutyl homologues (Table 2). This difference between the two series suggests that more than one dealkylation system may be present in liver microsomes.

TABLE 2. DEALKYLATION OF VARIOUS N-ALKYL DERIVATIVES OF 4-AMINOANTIPYRINE AND ANILINE

4-Aminoantipyrine derivatives		Aniline derivatives	
Alkyl groups	4-Aminoantipyrine formed (μmoles)	Alkyl groups	Aniline formed (μmoles)
CH_3—	1·26	CH_3—	0·98
$(CH_3)_2$—	0·34	$(CH_3)_2$—	0·90
C_2H_5—	0·32	C_2H_5—	0·96
$(C_2H_5)_2$—	0·19	$(C_2H_5)_2$—	—
C_4H_9—	0·34	C_4H_9)—	0·86
$(C_4H_9)_2$—	0·10	$(C_4H_9)_2$—	—

Lyophilized microsomes equivalent to 1 g of rabbit liver were incubated with 5 μmoles substrate, as described in "Methods". Glucose and glucose dehydrogenase were used as the TPNH-generating system.

TABLE 3. INHIBITION OF THE MICROSOMAL DEALKYLATION BY SKF 525-A

Substrate	Inhibition (%)
Amidopyrine (dimethyl-4-aminoantipyrine)	60
Monomethyl-4-aminoantipyrine	40
Ephedrine	45
Pethidine	50
N-Methylaniline	0
N-Ethylaniline	0
N-Butylaniline	0
N : N-Dimethylaniline	0
Monoethyl-4-aminoantipyrine	0
Monobutyl-4-aminoantipyrine	0
Mepacrine	0

Microsomes prepared from 1 g liver were incubated with 5 μmoles substrate as described under "Methods" with and without the addition of 4×10^{-4} M SKF 525-A*. Glucose-6-phosphate and the soluble fraction of the cell used as the TPNH-generating system.

* SKF 525-A at a concentration of 5×10^{-3} M induces partial denaturation of proteins.

Experiments with the non-competitive inhibitory agent, SKF 525-A,[10] provided more definite evidence for more than one dealkylation system in microsomes. This compound, at a concentration of 1×10^{-4} M, inhibited the dealkylation of some N-alkylamines but not of others. For example, it inhibited the demethylation of dimethyl-4-aminoantipyrine (amidopyrine), MMAP, ephedrine and pethidine but did not affect the demethylation of methylaniline. Furthermore, SKF 525-A did not inhibit the dealkylation of a number of ethyl- and butylalkylamines, including homologues of 4-aminoantipyrine (Table 3).

Other results, indicative of more than one dealkylation system, were obtained by comparing dealkylation rates of various drugs by rabbit and guinea pig microsomes. Ratios of dealkylation rates for various compounds in the two species are presented in Table 4. The ratios for MMAP and pethidine were the same, whereas the ratio for

TABLE 4. DEALKYLATION OF VARIOUS SUBSTRATES BY MICROSOMES OF RABBIT AND AND GUINEA PIGS

Species	Ratio of dealkylation rates		
	MMAP*/Pethidine	MMAP/MEAP†	MMAP/Methylaniline
Rabbit	1 : 1	5 : 1	4 : 1
Guinea pig	1 : 1	1 : 1	1 : 1

Microsomes prepared from 1 g liver were incubated with 5 μmoles substrate as described under "Methods". Glucose-6-phosphate and the soluble fraction were used as the TPNH-generating system.

* MMAP, Monomethyl-4-aminoantipyrine

† MEAP, Monoethyl-4-aminoantipyrine.

MMAP and its ethyl homologue was higher in rabbits than guinea pig. These results suggest that the enzyme systems involved in the dealkylation of the methyl and ethyl derivatives of 4-aminoantipyrine may be different. Similar studies indicate that methylaniline and MMAP also may be dealkylated by different enzymes.

TABLE 5. DEALKYLATION OF SARCOSINE BY RABBIT LIVER MITOCHONDRIA

Incubation system	Formaldehyde formed (μmoles/hr per g tissue)
Mitochondria	1·50
Mitochondria + 1×10^{-3} M CN^-	0·70
Dialysed mitochondria	0·10
Dialysed mitochondria + TPNH-generating system*	0·19
Dialysed mitochondria + DPNH-generating system*	0·13
Dialysed mitochondria + 1×10^{-4} M cytochrome *c*	0·62

* Glucose dehydrogenase with 0·2 μmole of coenzyme was used as the generating system.

Mitochondria from 2·5 g of liver were dialysed for 24 hr against 0·1 M phosphate buffer (pH 7·4) and then incubated for 1 hr at 37°C with 5 μmoles of sarcosine and additions as indicated, in a final volume of 5 ml of 0·1 M phosphate buffer.

Demethylation of sarcosine by mitochondria

Mackenzie[4] has reported that dimethylglycine and monomethylglycine (sarcosine) are demethylated to glycine and formaldehyde by two different enzyme systems localized in liver mitochondria. Since microsomal systems do not dealkylate these methylamino acids (Table 1), it was of interest to compare the properties of the microsomal and the mitochondrial enzyme systems. Rabbit liver mitochondria, prepared as described by Mackenzie[4], readily demethylated sarcosine. Activity was lost on dialysis against phosphate buffer for 24 hr. The addition of flavine adenine dinucleotide (FAD) (50 μg), TPN (200 μg), DPN (200 μg), DPNH (0·2 μmole) and TPNH (0·2 μmoles) failed to restore the activity, but considerable activity was restored on the addition of cytochrome-*c* (10^{-4} M) (Table 5). This observation is of particular interest since cytochrome-*c* inhibits the dealkylation of foreign compounds by microsomes. In contrast, cyanide which does not affect microsomal dealkylation,[11] markedly inhibited the demethylation of sarcosine.

When amidopyrine or MMAP was incubated with the mitochondrial preparation, no demethylation was observed under conditions in which sarcosine was readily demethylated.

Moritani *et al.*[5] have demonstrated that an acetone powder extract of rabbit liver or kidney demethylates N-methyl-1-histidine and other methylamino acids, but does not act on sarcosine or dimethylglycine. These workers have postulated that the methylamino acids undergo dehydrogenation by the action of a FAD-flavoprotein. On incubation of amidopyrine and MMAP with acetone powder extracts of rabbit liver and kidney, these compounds were not demethylated under conditions in which N-methylhistidine was readily demethylated.

DISCUSSION

A diversity of foreign alkylamines are oxidatively dealkylated by enzyme systems in liver microsomes. These enzymes seem to have an extraordinary degree of nonspecificity. However, more than one dealkylation system may be present in rabbit microsomes. For example, the enzyme system that removes methyl groups from monomethyl-4-aminoantipyrine (MMAP) may differ from that which removes higher alkyl groups from homologues of 4-aminoantipyrine. β-Dimethylaminoethyl diphenylpropylacetate (SKF 525-A), a non-competitive inhibitor, antagonizes the demethylation of MMAP, pethidine and a number of other methylated amines, but does not interfere with the dealkylation of the ethyl and butyl homologues of MMAP. Moreover, the relative rates of dealkylation in rabbit and guinea pig microsomes are the same for MMAP and pethidine, but different for MMAP and its ethyl homologue. Of particular interest is the evidence that the enzyme system that demethylates methylaniline may differ from that which removes the methyl groups from a number of other methylamines. Axelrod[3] has also reported evidence for more than one demethylation system present in microsomes. Despite the probability that more than one dealkylation system is present in microsomes, it is likely that the dealkylation of a host of foreign alkylamine compounds is achieved by relatively few enzymes.

A traditional belief holds that drugs undergo chemical transformation because their structures are similar to those of substrates in intermediary metabolism. It is noteworthy, therefore, that normally occurring N-alkylamines, such as sarcosine, dimethylglycine, dimethylaminoethanol, choline, creatine and epinephrine are not

demethylated by liver microsomes, though the demethylation of sarcosine and dimethylglycine is accomplished by highly specific systems in mitochondria.[4] A similar situation exists for the hydroxylation of aromatic compounds. Despite the non-specificity of the microsomal hydroxylating system with respect to foreign compounds, it does not promote the hydroxylation of L-phenylalanine, L-tryptophane, kynurenine and anthranilic acid, naturally occurring compounds which are hydroxylated by quite specific systems localized in other parts of the liver cell.[12]

The failure of liver microsomes to act on these normally occurring substrates is surprising in view of the unusually broad range of foreign compounds which they metabolize. Some insight into this apparent paradox is provided by a comparison of the metabolism of a series of foreign N-alkylamines. Only those compounds that have a high chloroform to water partition ratio are oxidized by rabbit microsomes *in vitro* (Table 1). This suggests that the microsomal oxidative systems are protected by a lipoid barrier which only fat soluble substances can penetrate. The evidence for this hypothesis is strengthened by the results with the series, caffeine, theophylline and theobromine; only caffeine, which has a relatively high lipid solubility, is demethylated by microsomes. An alternative explanation may be that the active sites on the microsomal enzymes can interact only with non-polar substances. It should be also noted that the afore-mentioned normal substrates which are not hydroxylated by liver microsomes, are also poorly lipid soluble. Other examples of the importance of lipid solubility are found in substrates of the deamination enzyme present in rabbit microsomes, but absent in a number of other mammalian species.[13] This enzyme deaminates the lipid soluble substances amphetamine, methamphetamine and ephedrine, but does not act on substances like tyramine or serotonin,[13] which are substrates of monoamine oxidase. Older studies have indicated that monoamine oxidase in rabbits, but not in other species, deaminates mescaline.[14] This observation can be explained if mescaline, a highly lipid soluble compound, is metabolized by rabbit liver microsomes.

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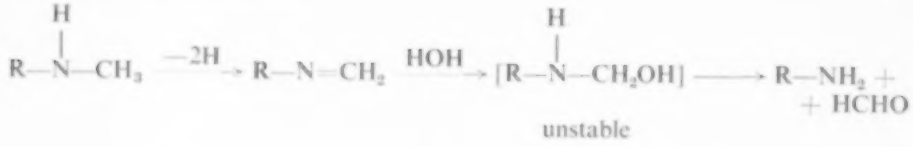
It is of particular interest that the dealkylation of foreign compounds is achieved by quite a different mechanism than that which acts on sarcosine. Dealkylation by the microsomal system requires TPNH and involves activation of oxygen; similar requirements have been noted for the oxidative mechanisms in microsomes that hydroxylate aromatic rings and aliphatic side chains, cleave ethers, deaminate alkylamines and oxidize sulphur-containing compounds to sulphoxides.[15] It has been suggested that a common step in microsomal oxidation may be the formation of an intermediate "hydroxyl" donor, which, in conjunction with a number of non-specific enzymes, can transfer an hydroxyl group to an appropriate acceptor substrate.[15] Thus, dealkylation of a foreign N-alkylamine may be written as the direct substitution of a hydroxyl grouping for a hydrogen.

$$R{-}NHCH_3 \xrightarrow{[OH]} \underset{\text{unstable}}{[R{-}NHCH_2OH]} \longrightarrow RNH_2 + HCHO$$

The mitochondrial enzyme system which acts on sarcosine does not require TPNH and unlike the microsomal systems it is blocked by cyanide. It appears to be a de-

hydrogenase linked with the cytochrome system. Thus, the dealkylation of sarcosine may be written as follows:



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THE ACCELERATING EFFECT OF NARCOTIC ANALGESICS ON THE HYDROLYSIS OF AROMATIC SUBSTRATES BY HUMAN PLASMA CHOLINESTERASE*

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Abstract—The activating effect of twelve narcotic analgesics on the hydrolysis of two aromatic substrates (procaine and benzoylcholine) by plasma cholinesterase was investigated. Allyl substitution decreased the accelerating effect. Oxidation of the alcoholic hydroxyl to a ketone at C_6 of the morphine molecule markedly increased the accelerating effect on the enzymic hydrolysis of procaine. This was further enhanced by the simultaneous substitution of H by OH at C_{14}. Some of the possible explanations of these accelerating mechanisms are discussed.

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PREVIOUS communications indicated that various alkylamines enhance the hydrolysis of aromatic and heterocyclic substrates by plasma cholinesterase.[1, 2, 3, 4] Furthermore, during an investigation of the inhibitory effect of narcotic analgesics on human cholinesterases,[5] it was observed that most of these compounds, in relatively low concentrations, accelerate the enzymic hydrolysis of benzoylcholine (BeCh) and procaine. Hazard[6] previously reported on the effect of dihydrohydroxycodeinone on the hydrolysis of procaine; Cannava[7] described the similar activity of several morphine derivatives; and Fraser[8] investigated the influence of morphine and nalorphine on the hydrolysis of BeCh by plasma cholinesterase. These findings, as well as our preliminary results,[5] stimulated a systematic study of the problem. Consequently, investigation of the accelerating effect of twelve narcotic analgesics on the hydrolysis of BeCh and procaine by a purified human plasma cholinesterase concentrate, "Cholase",* was undertaken.

MATERIALS AND METHODS

The accelerating effect of morphine phosphate, nalorphine hydrochloride (*N*-allyl-normorphine hydrochloride), levorphan tartrate (3-hydroxy-*N*-methylmorphinan tartrate), levallorphan tartrate (3-hydroxy-*N*-allylmorphinan tartrate), dihydromorphinone hydrochloride, 14-hydroxydihydromorphinone hydrochloride, dihydrocodeine bitartrate, dihydrocodeinone bitartrate, dihydrohydroxycodeinone hydrochloride, meperidine hydrochloride (*N*-methyl-4-phenyl-4-carbethoxypiperidine

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* Cholase is the solution of a human plasma cholinesterase concentrate prepared similarly to Harvard fraction IV-6-3. One millilitre of Cholase contains 35 mg of protein. Its activity with acetylcholine substrate corresponds to about 160 ml fresh, pooled, heparinized human plasma.

hydrochloride), alphaprodine hydrochloride (DL-α-1 : 3-dimethyl-4-phenyl-4-propionoxypiperidine hydrochloride), and RO2-7113 (DL-1-methyl-3-allyl-4-phenyl-4-propionoxypiperidine hydrochloride) were investigated. The substrates used were procaine hydrochloride and BeCh chloride. In a few experiments, PDMI-666 (2-diethylaminoethanol benzoic ester hydrochloride), PDMI-667 (triethyl (2-hydroxyethyl) ammonium bromide, *p*-aminobenzoic ester hydrobromide), PDMI-669 (triethyl (2-hydroxyethyl) ammonium iodide, benzoic ester) were also employed.

The hydrolysis of procaine and PDMI-667 was measured by a modification of Kalow's[9] u.v spectrophotometric method in a Beckman model DU spectrophotometer. The substrate concentration in these experiments was 5×10^{-5} M. The hydrolysis of BeCh, PDMI-666 and 669 was assayed by Ammon's[10] modification of Warburg's manometric technique. The substrate concentration in the manometric studies was 2×10^{-3} M. In the u.v. spectrophotometric method a phosphate buffer of pH 7·4 usually was employed. In some parallel experiments borax–boric acid or pyrophosphate buffers of pH 7·4 and 9·0 were also used. None of the buffers contained any divalent cations. In the Warburg manometric studies the buffer system consisted of a 0·025 M sodium bicarbonate solution equilibrated with 5 per cent CO_2 in nitrogen gas. In both methods the temperature was kept constant at 37°C. The analgesics, in from four to eight different concentrations, were added to the systems 15–20 min before the addition of the substrates. All experiments were done in duplicate, on at least two different occasions. The results were evaluated by plotting the negative logarithms of the molar concentrations of analgesics against change in activity expressed as percentage of control value. The source of enzyme was Cholase; dilution was 1 : 750 (v/v) in the procaine and 1 : 8000 (v/v) in the BeCh experiments. In some control studies heparinized human plasma was used with similar results. It is not known whether plasma or Cholase contain one or more enzymes which can hydrolyse the substrates used. For the sake of brevity, it will be assumed that only one enzyme was involved in the mechanisms studied.

Table 1. Acceleration of the enzymic hydrolysis of procaine and benzoylcholine by narcotics

Compounds	Procaine		Benzoylcholine	
	Concentration (M)	Maximum acceleration (%)	Concentration (M)	Maximum acceleration (%)
Morphine phosphate	$5\cdot0\times10^{-4}$	96	$1\cdot0\times10^{-3}$	138
Nalorphine HCl	—	—	$3\cdot3\times10^{-4}$	34
Levorphan tartrate	$2\cdot0\times10^{-5}$	60	$1\cdot0\times10^{-4}$	62
Levallorphan tartrate	$1\cdot0\times10^{-5}$	49	$3\cdot3\times10^{-5}$	15
Dihydrocodeine bitartrate	$6\cdot5\times10^{-5}$	70	$3\cdot3\times10^{-4}$	111
Dihydrocodeinone bitartrate	$2\cdot5\times10^{-4}$	214	$1\cdot0\times10^{-4}$	55
Dihydromorphinone HCl	$1\cdot0\times10^{-3}$	254	$3\cdot3\times10^{-4}$	103
Dihydrohydroxycodeinone HCl	$1\cdot0\times10^{-3}$	327	$3\cdot3\times10^{-4}$	65
14-Hydroxydihydromorphinone HCl	$1\cdot0\times10^{-3}$	350	$1\cdot0\times10^{-3}$	84
Meperidine	—	—	$1\cdot0\times10^{-3}$	52
Alphaprodine	$5\cdot0\times10^{-5}$	28	$5\cdot0\times10^{-4}$	42
RO 2-7113	—	—	$1\cdot0\times10^{-4}$	7

RESULTS AND DISCUSSION

The experimental findings are summarized in Table 1 and in Figs. 1, 2 and 3. Figure 1 shows the effect of morphine, levorphan and their allyl derivatives on the enzymic hydrolysis of procaine and BeCh. It is evident that the allyl derivatives were less effective accelerators and, as previously reported,[11, 14] better inhibitors of the enzyme than were the parent compounds.

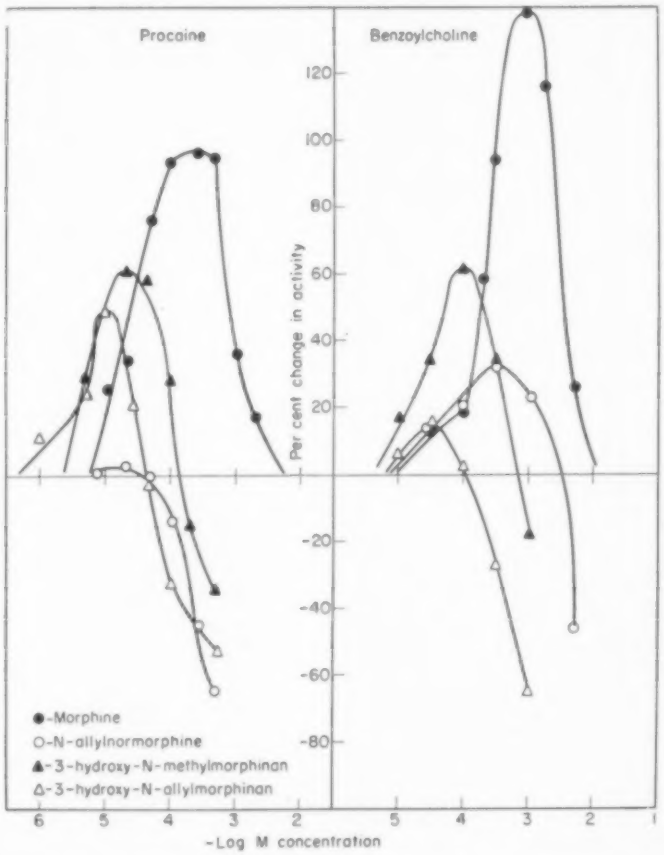

FIG. 1. Acceleration of the enzymic hydrolysis of procaine and BeCh by morphine, *N*-allylnormorphine, 3-hydroxy-*N*-methylmorphinan, and 3-hydroxy-*N*-allylmorphinan.

All morphinone and codeinone derivatives (see Fig. 2) enhanced the enzymic hydrolysis of procaine more than that of BeCh. The acceleration of the hydrolysis of procaine by plasma cholinesterase was greatest with 14-hydroxydihydromorphinone (350 per cent), dihydrohydroxycodeinone (327 per cent), and dihydromorphinone (254 per cent). In contrast to this, the order of the accelerating effect on the enzymic hydrolysis of BeCh was: morphine 138 per cent, dihydrocodeine 111 per cent, and dihydromorphinone 103 per cent.

The accelerating effect of meperidine, alphaprodine, or of RO2-7113 was considerably less than those of the various morphine derivatives (Fig. 3).

The effects of morphine and levorphan on the enzymic hydrolysis of PDMI-667, the quaternary triethyl derivative of procaine, were similar to those exerted on that of the parent compound. On the other hand, substitution of the methyl by ethyl groups on the quaternary-*N* of BeCh (PDMI-669), or removal of the *p*-amino group of procaine (PDMI-666), abolished the accelerating effect on these two substrates. Increasing the concentration of BeCh from 2×10^{-3} M to 2×10^{-2} M and leaving the concentration of morphine (10^{-3} M) unchanged resulted in a marked decrease (138 to 50 per cent) in the activity of this compound.

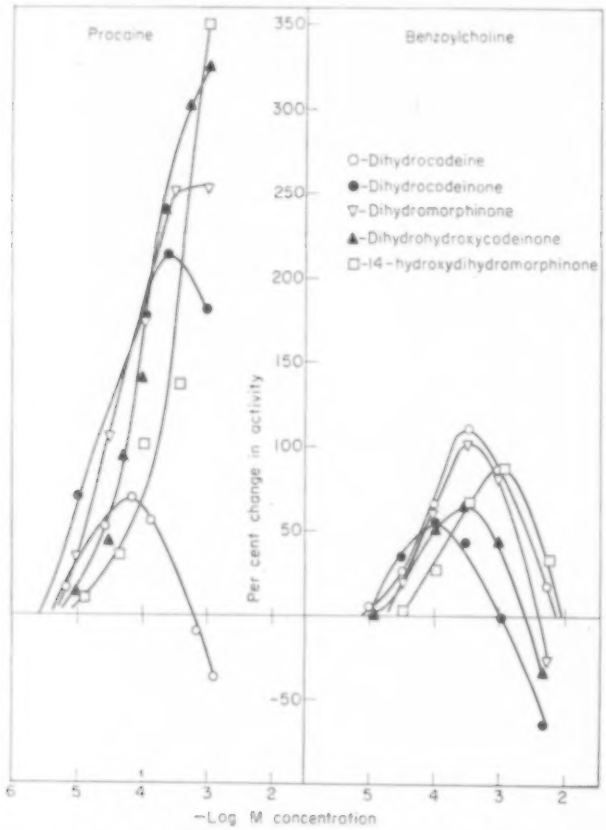

FIG. 2. Acceleration of the enzymic hydrolysis of procaine and BeCh by dihydrocodeine, dihydrocodeinone, dihydromorphinone, dihydrohydroxycodeinone, and 14-hydroxydihydromorphinone.

Morphine, levorphan and dihydrocodeine had a similar effect on the hydrolysis of BeCh when, instead of Cholase, heparinized human plasma was used. Investigation of the effect of morphine and levorphan on the hydrolysis of BeCh by horse serum *in vitro* revealed that morphine accelerated both horse and human plasma cholinesterase activity to the same extent. In contrast to this, levorphan did not accelerate the activity of the horse enzyme.

Examination of the experimental findings indicates certain relationships between the structures of the compounds used and their activity *in vitro*. In general, as first

shown by Fraser[8] with nalorphine and morphine, the allyl derivatives of the opioid alkaloids (nalorphine and levallorphan) are less potent accelerators and more potent inhibitors than the parent compounds (morphine and levorphan) (see Fig. 1). No marked differences were found between the accelerating effects of morphine and dihydrocodeine on the enzymic hydrolysis of BeCh, and dihydrocodeine was even less effective than morphine with procaine substrate. On the other hand, oxidation of the alcoholic OH group to a ketone at C_6 (dihydrocodeinone or dihydromorphinone) (Fig. 4) caused an increase in the acceleration of the hydrolysis of procaine and a

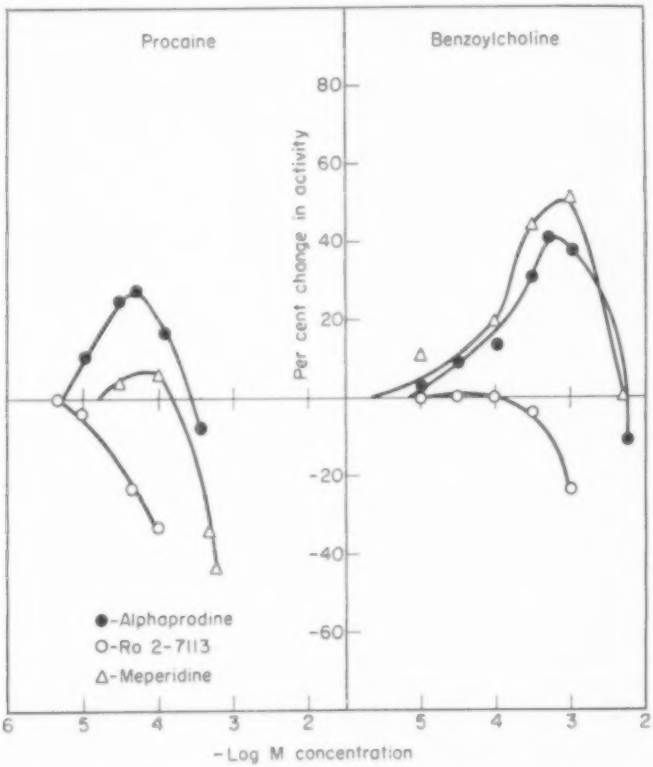

FIG. 3. Acceleration of the enzymic hydrolysis of procaine and BeCh by alphaprodine, RO 2-7113 and meperidine.

decrease of that of BeCh. Substitution of H by OH in the 14-position (dihydrohydroxycodeinone and 14-hydroxydihydromorphinone) further enhanced the breakdown of procaine.

It is of interest that all the changes, i.e. substitution of an alcoholic OH with a ketonic oxygen (= O) at C_6 and addition of an OH group at C_{14}, which increased the activity with procaine substrate, occurred in that part of the molecule of the morphine-type activators which is coplanar with the N atom. According to Barlow[12], this part of the molecule forms a 60–80° angle with the rest of the structure.

Although the accelerating effect of narcotic analgesics which are structurally different from morphine (meperidine and alphaprodine) is relatively weak, the

substitution of an allyl group (RO2-7113) further decreases this effect. Thus, while alphaprodine accelerates the hydrolysis of procaine and BeCh 28 per cent and 42 per cent, respectively, its allyl derivative RO2-7113, instead of accelerating, primarily inhibits the enzyme.

In general, the increase in the enzymic hydrolysis of procaine was considerably higher than that of BeCh. The value of this comparison is weakened, however, by the difference in the experimental conditions under which the hydrolysis of the two substrates was observed. Although the Michaelis constant (K_m) of both substrates

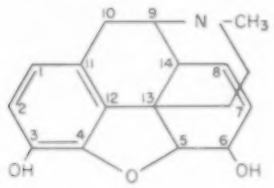

Fig. 4. The structural formula of morphine.

is about the same order of magnitude (10^{-6} M), for technical reasons the enzyme and substrate concentrations were markedly different in the two systems. Because of the relatively slow enzymic breakdown of procaine,[13] its hydrolysis had to be measured with an u.v. spectrophotometric method. With this method, the enzyme concentration was about eleven times higher and the substrate concentration forty times lower than in the Warburg manometric studies in which the enzymic hydrolysis of BeCh was measured. Although cleavage of the ester bond of BeCh can be assayed by an ultra-violet spectrophotometric method,[9] the compounds used in the present study interfered with the spectrophotometric determination of its hydrolysis.

Generally, the activating effect of the narcotic analgesics on the hydrolysis of procaine varied inversely with their inhibitory effect on the hydrolysis of acetylcholine.[14] Thus, for example, the relatively weak accelerators like nalorphine, levallorphan, and RO2-7113, were the best inhibitors, and the best activators like dihydrohydroxycodeinone, and 14-hydroxydihydromorphinone were the poorest inhibitors of the hydrolysis of acetylcholine by Cholase.

As already reported,[5] the hydrolysis of aliphatic substrates could not be accelerated, but only inhibited, by narcotic analgesics. This suggests that certain differences must exist between the modes of adsorption of the aliphatic and aromatic substrates to the surface of plasma cholinesterase. At present one can only speculate on the nature of these differences. The substrates used in the present experiments might be attracted to the surface of plasma cholinesterase by at least three different forces of attraction: (*a*) coulombic forces, between the N^+ and the anionic site of the enzyme;[15, 16] (*b*) a covalent bond, between the ester group and the esteratic site;[17] and (*c*) Van der Waal's forces, between the aromatic ring and an unspecified site on the enzyme. With aliphatic substrates, the Van der Waal's forces are less important. It is conceivable that aromatic substrates might be adsorbed, by virtue of attraction of their N^+, to the anionic site and by their benzene ring to other unspecified groups in a position which is unfavourable for the reaction between their ester group and the functionally important esteratic

site of the enzyme. Compounds that will prevent the adsorption of the N^+ of the aromatic substrates to the anionic site, or theoretically the adsorption of the benzene ring to unspecified sites, may facilitate the more favourable orientation of its ester group to the esteratic site. It was shown previously by Erdös *et al.*[3] that relatively simple alkylamines accelerate the enzymic hydrolysis of aromatic, but not that of aliphatic substrates. The compounds used in the present study, at the pH of the experimental methods, possess both N^+ and bulky ring systems. Therefore, they may be capable of competing with both the N^+ of the aromatic substrates for the anionic sites, and with their benzene ring for the unspecified sites. It is of interest that, according to Beckett[18], the surface of the analgesic receptor also contains an anionic site.

To test the validity of the importance of the N^+ on the accelerating mechanism, the effect of morphine (10^{-4} M), levorphan (3×10^{-5} M), and tetraethyl ammonium (10^{-3} M) on the hydrolysis of PDMI-667 by plasma cholinesterase was investigated at pH 7·4 and 9·0. The enzyme and substrate concentrations were the same as in the procaine experiments. The reason for choosing PDMI-667 instead of procaine was that while the enzymic hydrolysis of this quaternary-N compound is enhanced by the various activators similarly to that of procaine, it does not lose its cationic character at higher pH values. Since the pK_a of morphine is 8·05 and that of levorphan 8·18,[18] at pH 7·4, more than 80 per cent of their molecules are positively charged, whereas at pH 9·0, the percentage of the positive charge drops to less than 14 per cent.[19] The effect of changing pH on the acceleration was studied with phosphate or boric acid–borate buffer at pH 7·4, and borate or pyrophosphate buffer at pH 9·0. There was a marked decrease in the accelerating effect of morphine when the pH was changed from 7·4 to 9·0. The acceleration at pH 7·4 was 61 per cent and at pH 9·0, 8 per cent. However, the influence of changing the pH on the accelerating effect of levorphan was less marked, 56 per cent at pH 7·4, and 33 per cent at pH 9·0, respectively. There was no marked change between the accelerating effect of tetraethylammonium at pH 7·4 (62 per cent) and at pH 9·0 (57 per cent). The ionization of this compound does not change between pH 7·4 and at pH 9·0. These findings and the experiments with horse serum cholinesterase further imply the the mechanism by which morphine and levorphan enhance the enzyme activity might be different.

It is improbable that the findings reported would have any immediate practical implications. The concentrations of narcotic analgesics, as used in clinical practice, are unlikely to reach high enough levels in the liver and plasma to influence the enzymic breakdown of procaine. On the other hand, these observations have implications concerning the nature of the surface of cholinesterase, the forces involved in the adsorption of various substrates, and the binding of narcotic analgesics to this protein.

Undoubtedly, more work will be required before the mechanism of the accelerating effect of various compounds can be fully explained.*

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* The various compounds used in these studies were kindly supplied by the following individuals: nalorphine hydrochloride, Dr. F. K. Heath, Merck Sharp and Dohme Research Laboratories; levorphan tartrate, levallorphan tartrate, alphaprodine hydrochloride, RO2-7113, and benzoylcholine, Dr. L. A. Pirk, Hoffmann-LaRoche, Inc.; dihydrocodeine bitartrate, Mr. H. O. Burks, Knoll Pharmaceutical Co.; dihydrohydroxycodeinone hydrochloride and 14-hydroxydihydromorphinone hydrochloride, Dr. M. J. Lewinstein, Endo Laboratories, Inc.; morphine phosphate, Mr. T. J. Dwulit, Mallincrodt Chemical Works; PDMI-66, -667 and -669, Dr. G. W. Moersch, Parke, Davis and Co.; Cholase, Dr. J. H. Hink, Cutter Laboratories; dihydromorphinone hydrochloride, meperidine hydrochloride, and dihydrocodeinone bitartrate, Sister M. Gonzales, Mercy Hospital.

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REACTION OF 6-MERCAPTOPURINE WITH INOSINE- AND GUANOSINE- 5′ -PHOSPHATE PYROPHOSPHORYLASE PURIFIED FROM *E. COLI.*

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(*Received* 20 *February* 1959)

Abstract—An enzyme has been purified from *E. coli* which, in a reaction with 5-phosphoribosylpyrophosphate (PRPP) and guanine, hypoxanthine or 6-mercaptopurine (6-MP), converts the purine bases to the corresponding 5′-nucleotides. Kinetic data demonstrate an affinity of 6-MP for the enzyme greater than that of guanine and equal to that of hypoxanthine.

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THE conversion of 6-mercaptopurine to the 5′-ribonucleotide through reaction with 5-phosphoribosyl-1-pyrophosphate (PRPP), catalysed by inosinic acid pyrophosphorylase purified from liver, has been described by Lukens and Herrington[1]. Way and Parks[2] have reported a similar reaction in a less purified liver enzyme system. The role of this transformation in the susceptibility and resistance of organisms to 6-mercaptopurine has been studied by Brockman *et al.*[3], who found that resistant lines of *Streptococcus faecalis* and of L-1210 leukaemia failed to metabolize the purine analogue to the 5′-nucleotide.

This paper describes the purification of inosine- and guanosine-5′-phosphate pyrophosphorylase from *Escherichia coli*, and the kinetics of the reaction of the enzyme with guanine, hypoxanthine and 6-mercaptopurine.

METHODS

The method of Kornberg *et al.*[4] for the preparation of PRPP was employed. Solutions of the compound were used after removal of protein and nucleotides with charcoal.[5] The purines, purine nucleotides and ribose-5-phosphate were commercial products of demonstrated chromatographic purity. Samples of 4-oxo- and 4-thio-pyrimido-(4, 5-D)-pyrimidine and 4-oxo- and 4-thio-pteridine were synthesized by Dr. H. G. Mautner[6] of this department. Xanthine oxidase was purchased from Worthington Chemical Corporation.

Purification of inosine- and guanosine-5′-phosphate pyrophosphorylase

E. coli, strain B, was grown for 10 hr in a salts and glucose medium[7] and harvested in the Sharples centrifuge. Cells (40 g) were ground at 4°C with 120 g of Alcoa A-303 alumina, extracted with 100 ml of a solution containing 0·001 M tris buffer (pH 7·4) and 0·01 M magnesium acetate and centrifuged at 8000 × *g* to remove alumina and debris. This solution was then centrifuged at 105,000 × *g* for 2 hr to obtain a clear supernatant fraction. From 40 g (wet weight) of cells, 70 ml of the supernatant fraction

were obtained. (These fractions were generously supplied by H. Felsenfeld.) Ribonucleoproteins were precipitated from the supernatant fluid by making the solution 0·05 M with respect to $MnCl_2$ and centrifuging at 4°C and 4000 × *g* for 30 min. The solution was then dialysed for 3 hr against distilled water. Isoelectric precipitation of the enzymic activity was achieved by addition of one-tenth volume of 20% potassium acetate (pH 5·0). The precipitate was collected by centrifugation at 4°C and 4000 × *g* for 30 min and dissolved in 10 ml of 0·05 M tris buffer (pH 8·0). Heat treatment of this solution at 60°C for 3 min, followed by centrifugation at 4000 × *g* at 4°C for 15 min, removed approximately 70 per cent of the protein without appreciably diminishing enzymic activity. Further purification of the enzyme was achieved by precipitation at 60% ammonium sulphate saturation at 4°C. The precipitate was collected by centrifugation and dissolved in 10 ml of distilled water. Residual ammonium sulphate was not removed by dialysis. At this stage the enzyme fraction contained about 0·1–0·3 mg protein per ml, represented about twentyfold purification of the MnCl-treated extract and, more importantly, contained no 5′-nucleotidase activity. The enzyme solution was stable for over 1 month when kept in the frozen state.

EXPERIMENTAL AND RESULTS

The spectrophotometric assays employed depend upon the difference between the u.v.-absorption spectra at pH 7·0 of guanine and guanosine-5′-phosphate (GMP), and 6-mercaptopurine (6-MP) and 6-mercaptopurineribosyl-5′-phosphate (6-MP-R5′P), as shown in Fig. 1. The conversion of hypoxanthine to inosinic acid (IMP) is

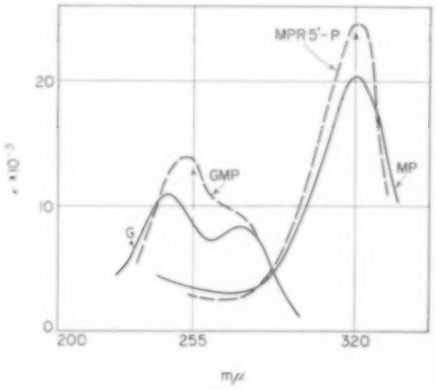

FIG. 1. The absorption spectra at pH 7·0 in 0·005 M phosphate buffer of guanine, GMP and 6-MP were determined on authentic samples and are in agreement with published values. The spectrum of 6-MP-R5′P at pH 7·0 is based upon the enzymic transformation of 6-MP to the nucleotide and determination of absorption characteristics of the incubation mixture upon completion of the reaction. This spectrum is in agreement with chromatographically isolated 6-MP-R5′P when molar absorptivity, *E*, is calculated on the basis of phosphate (see Table 1).

associated with a hyperchromic spectral shift, but this is too small to permit reliable kinetic data to be obtained. Consequently, the spectrophotometric assay for hypoxanthine employing xanthine oxidase[8,1] was used to follow the inosine-5′-phosphate pyrophosphorylase reaction, as shown in Fig. 2.

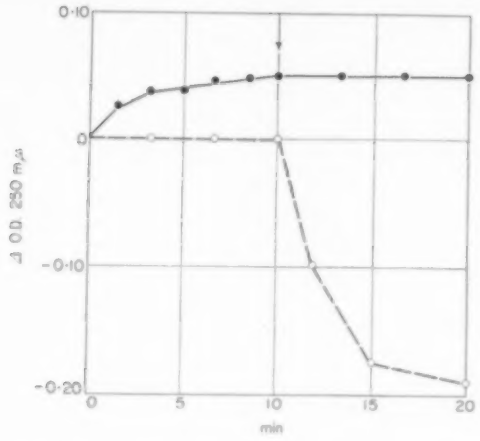

FIG. 2. The reaction of hypoxanthine with enzyme and PRPP. Incubation mixtures consisted of 0·05 μmole of hypoxanthine, 1·0 ml H_2O, 0·03 ml of 1 M sodium acetate, 0·04 ml 0·1 M $MgCl_2$ and 0·02 ml enzyme solution. To one cuvette 0·1 ml (0·1 μmole) PRPP was added (○ — — — — ○), and from the other it was omitted (● ——— ●). Absorption at 250 mμ was determined during the course of the incubation. After 10 min xanthine oxidase (XO) was added to both cuvettes. The decrease in absorption at 250 mμ in the cuvette containing no PRPP corresponds to the complete conversion of 0·05 μmole of hypoxanthine to uric acid. In the complete reaction mixture there was no decrease in absorption, a circumstance indicating the complete conversion of hypoxanthine to IMP.

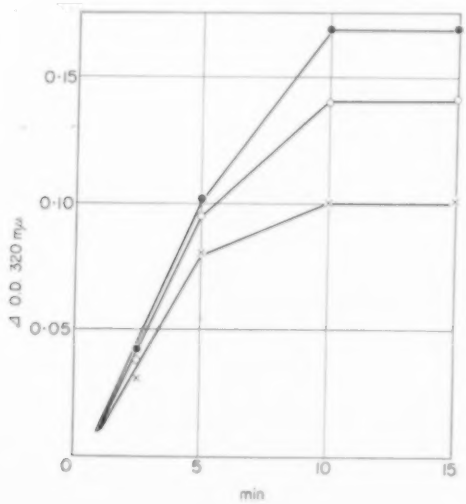

FIG. 3. The effect of the concentration of PRPP on the enzymic conversion of 6-MP to the 5′-nucleotide. Incubation mixtures consisted of 0·05 ml 6-MP (1 μmole/ml), 1·0 ml H_2O, 0·03 ml 1 M sodium acetate, 0·04 ml 0·1 M $MgCl_2$, 0·02 ml enzyme solution and 0·05 ml (×), or 0·075) ml (○) or 0·1 ml (●) of the enzymically prepared PRPP solution. Maximal rate and extent of the reaction occurred at the 0·1 ml level; therefore it is assumed that the concentration of PRPP is at least 0·5 mole/ml. The affinity constant of PRPP, based on these data, is between 10^{-4} and 10^{-5}M.

In Figs. 2, 3 and 4, spectrophotometric assays of nucleotide pyrophosphorylase with 5′-phosphoribosylpyrophosphate and the purine substrates hypoxanthine, 6-MP and guanine, respectively, are shown. The assays were conducted in silica cuvettes (1 ml) containing 0·05 ml purine (1 μmole/ml), 1·0 ml H_2O, 0·03 ml 1 M sodium acetate, 0·1 ml PRPP (0·05 to 0·1 μmole), 0·04 ml 0·1 M $MgCl_2$, and 0·02 ml enzyme solution; pH 7·2. As shown in Figs. 3 and 4, the rate and extent of the reaction are proportional to enzyme and PRPP concentration.

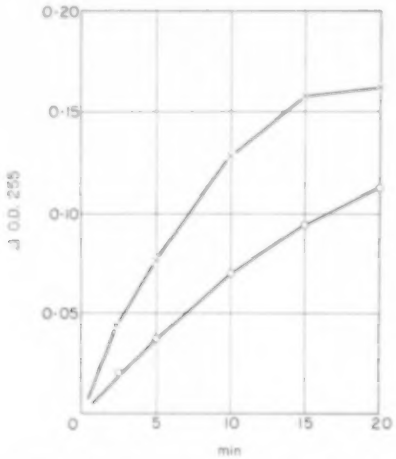

FIG. 4. The effect of enzyme concentration upon enzymic conversion of guanine to GMP. The protein concentration of the enzyme solution was 0·1 mg/ml; × = 0·02 ml of enzyme; ○ = 0·01 ml of enzyme. Incubation mixtures consisted of 0·05 ml guanine (1 μmole/ml), 1·0 ml H_2O, 0·03 ml, 1 M sodium acetate, 0·04 ml 0·1 M $MgCl_2$, 0·1 ml PRPP solution.

The enzymic activity is characterized by a broad pH optimum at 7·0–7·8 and, in agreement with previously published data[9] for nucleotide pyrophosphorylases, a requirement for Mg^{2+}. Inorganic pyrophosphate at a concentration of 2 μmoles/ml completely inhibited the enzyme, whereas orthophosphate at the same concentration caused less than 10 per cent inhibition. No pyrophosphorolysis of IMP, GMP or 6-MP-R5′P could be demonstrated by spectrophotometric assay or chromatography.

The affinity of guanine and 6-MP for the enzyme was determined from the data of Fig. 5, which show the influence of concentration of purine substrate on the rate of reaction. Employing the graphical plot method of Lineweaver and Burk for the determination of K_s, the following values were obtained: guanine = 5×10^{-4}; 6-mercaptopurine = 1×10^{-5} M. The higher affinity of 6-MP for the enzyme is reflected in the data of Fig. 6, which show that at a ratio of guanine to 6-MP of 5 : 3, the analogue is transformed to the 5′-nucleotide at the same rate as the 6-MP control in the absence of guanine. Since the conversion of the analogue to the 5′-nucleotide is associated with a hyperchromic shift at 320 μm, and the conversion of guanine to GMP produces a hyperchromic shift at 255 μm, the two reactions may be followed simultaneously. As shown in Fig. 6, the conversion of 0·05 μmole of guanine to GMP in the presence of 0·03 μmole of 6-MP takes place only after at least 75 per cent of the analogue present has been converted to 6-MP-R5′P. These data also show that the 6-MP-nucleotide is not an inhibitor of the enzymic conversion of guanine to GMP.

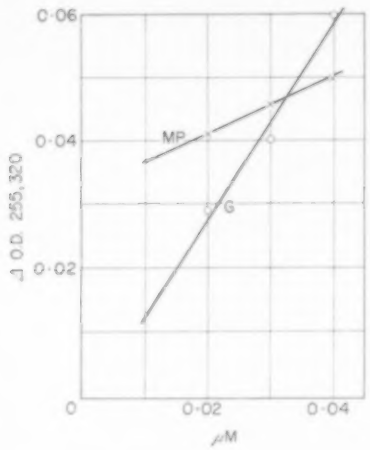

FIG. 5. The effect of concentration of purine substrate on initial reaction rates: guanine (○), changes in optical density followed at 255 mμ; 6-MP (×), changes in optical density followed at 320 mμ. Incubation mixtures consisted of from 0·02 to 0·04 μmole of purine, 1·0 ml H_2O, 0·03 ml, 1 M sodium acetate, 0·04 ml 0·1 M $MgCl_2$, 0·1 μmole PRPP in 0·1 ml and 0·02 ml of purified enzyme. Optical density changes for guanine were determined after incubation for 5 min and for 6-MP after 2 min. Initial reaction rates were linear during these intervals.

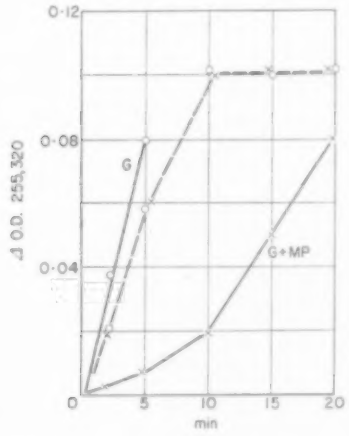

FIG. 6. Inhibition of guanylic acid synthesis by 6-MP. Incubation mixtures consisted of 0·1 μmole PRPP (0·1 ml), 1·0 ml H_2O, 0·03 ml 1 M sodium acetate, 0·04 ml 0·1 M $MgCl_2$ and the following purine additions: 0·05 μmole guanine, (○————○); 0·05 μmole guanine plus 0·03 μmole 6-MP (×————×). The conversion of guanine to the nucleotide is followed by increments of optical density at 255 mμ, represented by the solid line, and the conversion of mercaptopurine to the nucleotide by the increment at 320 mμ represented by the broken line (○— — — — ○), (×— — — — ×). The decrease in absorption at 255 mμ associated with the conversion of 0·03 μmole of 6-MP to the nucleotide is less than 0·01 and therefore does not interfere with simultaneous determination of reaction rates in incubation mixtures containing guanine and 6-MP in the above proportions.

The affinity constant of hypoxanthine for the enzyme is not readily derived from kinetic data because the spectrophotometric analysis of residual hypoxanthine by xanthine oxidase does not permit exact determination of initial reaction rates. However, as shown in Fig. 7, when equimolar amounts (0·05 μmole) of hypoxanthine and 6-MP are incubated with sufficient concentrations of enzyme and PRPP to bring about the transformation of 6-MP to the 5′-nucleotide at maximal rate, there is approximately 50 per cent inhibition of the rate and extent of the 6 MP reaction. This indicates that the affinity of hypoxanthine and the analogue for the enzyme are of the same order; $K_s = 1 \times 10^{-5}$ M. It is also clear that hypoxanthine and 6-MP compete for enzyme and PRPP.

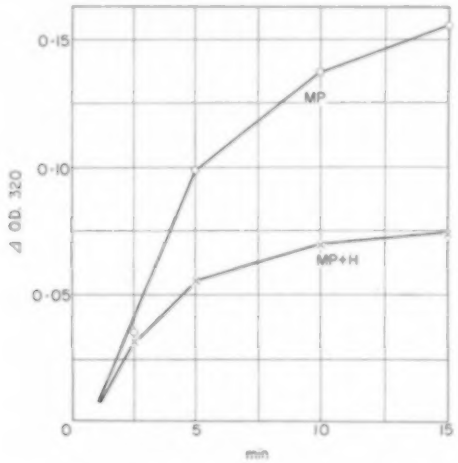

FIG. 7. The effect of an equimolar concentration of hypoxanthine on the enzymic conversion of 6-MP to the 5′-nucleotide. 6-MP, 0·05 μmole, alone, (○); hypoxanthine, 0·05 μmole plus 6-MP 0·05 μmole (×); PRPP addition was 0·1 ml, a concentration sufficient to convert 0·05 μmole of purine to the nucleotide at maximal rate in the presence of 0·02 ml of enzyme.

The substrate specificity of the enzyme prepared from *E. coli* corresponds to the liver enzyme purified by Lukens and Herrington.[1] Adenine, azaguanine, xanthine and 4-amino-5-imidazolecarboxamide are not substrates. The pyrimidopyrimidines are neither substrates nor inhibitors of the reaction, and the same is true of 4-oxo- and 4-thio-pteridine.

The earlier studies of Kornberg *et al.*[9] demonstrated that the equilibrium of the purine nucleotide pyrophosphorylase reaction is preponderantly in the direction of nucleotide synthesis. The enzyme purified from *E. coli* in this study completely transforms hypoxanthine, guanine and 6-MP to the 5′-nucleotides in the presence of an excess of PRPP. It was apparent from ion-exchange studies of the reaction products that the recovery of the 6-MP-nucleotide was low following an extended gradient separation in formic acid. As shown in Table 1, direct elution with the formate reagent yielded complete recovery of 6-MP-nucleotide. The nucleotide structure of the products isolated by ion-exchange chromatography was confirmed by determination of ribose, phosphate and the complete hydrolysis of nucleotide phosphorus to inorganic phosphorus by bull semen 5′-nucleotidase.[9]

TABLE 1.

Elution reagent	Tube no.	Nucleotide product GMP (μM)	IMP (μM)	6-MP-R5′P (μM)	Yield (%)
(A) 4 N formic acid (tubes 1–60)	24–25	0·51			100
0·2 M ammonium formate 4N formic acid (tubes 61–80)	36–38		0·49		99
(B) (tubes 1–20) 0·2 N ammonium formate 4 N formic acid	65–67			0·245*	59
	12–14			0·52	100

*No free 6-MP or other u.v.-absorbing peak was found.

Columns of Dowex-1, 200–400 mesh, 4 *x* cross-linkage, of 10 cm length and 0·9 cm diameter were converted to the formate form and, in (A) were eluted by developing a gradient with formic acid and formate ions in a 200 ml mixer of distilled water. Fractions of 8–9 ml were collected. In (B) direct elution with the ammonium formate–formic acid reagent was employed to minimize the period during which the 6-MP-nucleotide was on the column under acidic conditions. Fractions of 4–5 ml were collected.

Incubation mixtures consisted of 0·5 μmole of purine in 0·5 ml of H_2O adjusted to pH 7·0, 1·0 ml PRPP solution containing approximately 1·0 μmole, 0·05 ml 0·1 M $MgCl_2$, and 0·1 ml purified enzyme solution. After 30 min incubation the solutions were directly applied to columns.

The extinction coefficients employed in calculations were: GMP, $E_{260} = 12{\cdot}2 \times 10^3$; IMP, $E_{250} = 13{\cdot}9 \times 10^3$; 6-MP-R5′P, $E_{320} = 24 \times 10^3$.

DISCUSSION

Not all of the observations on the biological actions of 6-MP can be resolved in terms of its reactions at a single enzymic site. The results of the present study demonstrate an affinity of 6-MP for a purine nucleotide pyrophosphorylase greater than that of guanine and equal to that of hypoxanthine. Under certain restricted conditions, cells requiring hypoxanthine or guanine for purine nucleotide synthesis might be effectively inhibited by this mechanism. Organisms resistant to 6-MP have been found to be lacking in the capability to form 6-MP ribonucleotide.[3] This has been interpreted to mean that 6-MP exerts its antimetabolite activity after transformation to the nucleotide. The possibility remains that the failure to form 6-MP nucleotide in resistant cells is a consequence of the deletion of the enzymic mechanism for converting guanine and hypoxanthine to nucleotides through the pyrophosphorylase reaction. An active site for 6-MP inhibition might thus be removed.

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ACTION OF SOME PHENOTHIAZINE DERIVATIVES ON THE RESPIRATORY CHAIN

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Abstract—Several phenothiazine tranquillizers and their derivatives have been tested against the respiration of liver mitochondria. Two actions have been found: Competitive inhibition of cytochrome oxidase and inhibition of DPNH–cytochrome-*c* reductase. The latter is manifested only in phosphorylating systems. There is some correlation between the effect on DPNH–cytochrome-*c* reductase and tranquillizing ability. Thus, chlorpromazine inhibits 90 per cent at 2×10^{-4}M, at which concentration its sulphoxide has no effect. However, anomalies occur: acetylpromazine has little effect, but is a potent tranquillizer, and conversely, the primary amine of chlorpromazine is a powerful inhibitor, but is not a good tranquillizer.

Previous workers[1–3] have made scattered observations on the activity of chlorpromazine against the respiration of tissue homogenates. In addition, Lindan *et al.*[4,5] have made observations on the effect of this substance on the respiration of brain slices. The latter workers showed that chlorpromazine inhibited the potassium-activated respiration of brain slices, but not the resting oxygen uptake. The remainder of the results with cell-free preparations have been somewhat conflicting, the most constant finding being some degree of inhibition of oxidative phosphorylation. We have carried out detailed studies of the mode of action of chlorpromazine on the respiratory chain[6,8] and have shown two sites of action: One, an inhibitory effect upon cytochrome oxidase, the second, an inhibition of DPNH–cytochrome-*c* reductase. The present paper extends this work to other members of the phenothiazine group, and shows that some correlation exists between tranquillizing ability and activity of the compounds in inhibiting electron transport. However, this is not complete; at least two substances behave in an anomalous manner.

METHODS

Animals. Wistar albino rats were used throughout. They were fed the M.R.C. 41 diet *ad lib.*, were of either sex and were of 150–200 g body weight.

Chemicals. Nucleotides and cytochrome-*c* were obtained from Sigma Chemical Co., St. Louis, Mo.

Phenothiazines: Chlorpromazine hydrochloride, diethazine hydrochloride and compazine (prochlorperazine) methane sulphonate were gifts from May & Baker Ltd., Dagenham, Essex; chlorpromazine sulphoxide was a gift from Smith, Kline & French Labs., as were promethazine hydrochloride; promazine; the primary amine of chlorpromazine; stelazine and the sulphoxide of compazine. Mepazine hydrochloride was

*Stothert Research Fellow of the Royal Society.

obtained from W. R. Warner. Acetylpromazine was a gift of Dr. L. Golberg, Bengers Ltd., Holmes Chapel, Cheshire.

Enzyme preparations. Mitochondria from rat liver were prepared by the method of Schneider[9], in 0·25 M sucrose. Microsomes were isolated by centrifugation of the 0·25 M sucrose suspension at 105,000 × *g* for 30 min on the Spinco Model L preparative ultracentrifuge. When β-hydroxybutyrate was used as substrate, mitochondria were pretreated in 0·075 M sucrose for 15 min at 0°C. This was necessary in order to permit access of the inhibitor, since mitochondria in 0·25 M sucrose were less sensitive to the inhibitor. When succinate was used as substrate, mitochondria were pre-treated in 0·25 M sucrose–0·02 M inorganic orthophosphate buffer, pH 7·2 for 10 min at 30°C. This procedure depletes the mitochondria of their pyridine nucleotides and permits the study of the one-step oxidation of succinate. Mitochondria in water were kept at 0°C for 45 min before use.

Determinations. Cytochrome oxidase was studied in the following medium: Ascorbic acid, neutralized just before use to pH 7·2, 0·01 M, ethylene diamine tetra-acetate (versene) 0·001 M, sodium inorganic orthophosphate buffer pH 7·2, 0·017 M, and cytochrome *c* at the concentrations given in the tables. Mitochondria were added in 0·5 ml of a 5 per cent suspension in water. Final volume 3·0 ml. Temperature 38°C. Gas phase air.

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Oxidative phosphorylation was studied in the following medium: adenosine triphosphate (ATP) 0·0017 M, $MgSO_4$ 0·0067 M, KCl 0·025 M, cytochrome *c* 2×10^{-5} M, inorganic orthophosphate buffer pH 7·2, 0·01 M. Sodium hydroxybutyrate when present 0·01 M, sodium succinate when present 0·01 M, ascorbic acid when present 0·01 M, glucose 0·03 M, hexokinase 0·05 ml of a solution purified to stage 3 as described by Berger *et al.*[10] Mitochondria were added in 0·5 ml of suspension (details in tables) equivalent to 1·0 mg N, for β-OH-butyrate oxidation, and equivalent to 0·3 mg N for succinate oxidation. Final volume 3·0 ml. Temperature 38°C. Gas phase air. Standard manometric technique was used.

The reduction of cytochrome-*c* by β-hydroxybutyrate was followed in the system: sodium β-hydroxybutyrate 0·033 M, cytochrome *c* 4×10^{-5} M, sodium inorganic orthophosphate buffer pH 7·2, 0·033 M, sodium cyanide 0·0001 M, versene 0·001 M. Mitochondria equivalent to 0·004 mg N were added in a volume of 0·1 ml of water or 0·075 M sucrose as indicated in the tables. Final volume 3·0 ml. Temperature 20°C. When water-treated mitochondria were used it was necessary to add diphosphopyridine nucleotide 0·0003 M to obtain reduction of cytochrome-*c*. The reduction of cytochrome-*c* was measured by determining the increase in absorption at 550 mμ with a Unicam SP 500 spectrophotometer, using cuvettes with a 1 cm light path. The DPNH–cytochrome *c* reductase of the microsomes was determined using the same system, except that reduced diphosphopyridine nucleotide (DPNH) 0·00067 M replaced sodium β-hydroxybutyrate.

RESULTS

1. *Effects on cytochrome oxidase*

In a previous paper[7] we have shown that chlorpromazxine inhibited cytochrome oxidase, and that this inhibition was relieved by addition of extra cytochrome *c*. Table 1 shows that many of the phenothiazines tested are also inhibitors of cytochrome

Table 1. Effect of phenothiazines on cytochrome oxidase

Compound	Concentration (M)	% Inhibition of oxygen uptake Cytochrome-*c* 2×10^{-5}M	Cytochrome-*c* $6{\cdot}6 \times 10^{-5}$M
Chlorpromazine	10^{-4}	50	nil
	2×10^{-4}	89	68
Chlorpromazine sulphoxide	4×10^{-4}	5	nil
Promethazine	4×10^{-4}	24	nil
Diethazine	4×10^{-4}	76	63
Mepazine	4×10^{-4}	98	80
Acetyl promazine	2×10^{-4}	nil	nil
Primary amine of chlorpromazine	10^{-4}	77	65
	2×10^{-4}	94	—
Compazine	10^{-4}	nil	nil
	2×10^{-4}	22	10
Compazine sulphoxide	2×10^{-4}	5	nil
Stelazine	10^{-4}	90	72
Promazine	10^{-4}	58	nil
	2×10^{-4}	67	—

The components of the system were: ascorbic acid (neutralized just before use to pH 7·2), 0·01 M; versene, 0·001 M; sodium inorganic orthophosphate buffer, pH 7·2, 0·017 M; cytochorome-*c* as indicated in the table. Mitochondria in 0·5 ml of water equivalent to 0·1 mg N. Final volume 3·0 ml. Gas phase air and temperature 38°C. Readings were commenced after 7 min thermal equilibration and continued for 10 min, during which time they were linear. The control rate at 2×10^{-5}M cytochrome-*c* was 61 $\pm$ 3 μl O_2 taken up in 10 min; the control rate at $6{\cdot}6 \times 10^{-5}$M cytochrome-*c* was 76 $\pm$ 3 μl O_2 taken up in 10 min. The percentage inhibitions quoted are the mean of at least six determinations; the error being $\pm$5 per cent.

oxidase. The inhibitions when present are relieved somewhat by tripling the concentration of cytochrome-*c*. Table 2 shows a more detailed study of this point with chlorpromazine. It shows that the inhibition of cytochrome oxidase can be reversed by increasing the cytochrome-*c* concentration. We have shown[7] that this effect is due to competition between cytochrome-*c* and the drug for an active site on the enzyme concerned. Non-specific binding of chlorpromazine by cytochrome-*c* is unlikely.

Examination of the table shows some correlation between inhibition of cytochrome oxidase and tranquillizing ability. For example, chlorpromazine is a powerful inhibitor, whereas its sulphoxide (a metabolic detoxication product) is not. Similarly, promethazine and the sulphoxide of compazine are also relatively inactive. Stelazine and the primary amine of chlorpromazine are potent inhibitors, but acetylpromazine

Table 2. Effect of cytochrome-*c* concentration on chlorpromazine inhibition of cytochrome oxidase

Concentration of cytochrome-*c*, (M)	% Inhibition
2×10^{-5}	84
$6{\cdot}7 \times 10^{-5}$	68
$1{\cdot}33 \times 10^{-4}$	34
2×10^{-4}	18

Conditions of the experiment as in Table 1. Chlorpromazine concentration, 2×10^{-4} M. The percentage inhibitions quoted are representative of six similar experiments.

is not. Since the primary amine is not a good tranquillizer while acetylpromazine is, it would appear that the correlation is incomplete.

2. *Effect on mitochondrial DPNH–cytochrome-c reductase*

We have shown previously[8] that in addition to its action on cytochrome oxidase, chlorpromazine inhibits electron transport between DPNH and cytochrome-*c*. This inhibition apparently occurs only in mitochondria with an intact phosphorylation

TABLE 3. EFFECT OF PHENOTHIAZINE ON REDUCTION OF CYTOCHROME-*c* BY β-HYDROXYBUTYRATE

Compound	Concentration (M)	% Inhibition	
		Mitochondria in 0·075 M sucrose	Mitochondria in water
Chlorpromazine	10^{-4}	50	nil
Chlorpromazine	2×10^{-4}	90	12
Chlorpromazine sulphoxide	2×10^{-4}	15	nil
Promethazine	4×10^{-4}	50	nil
Diethazine	4×10^{-4}	22	nil
Mepazine	4×10^{-4}	80	nil
Acetyl promazine	2×10^{-4}	25	nil
Stelazine	10^{-4}	95	43
Compazine	10^{-4}	60	nil
Compazine sulphoxide	10^{-4}	nil	nil
Promazine	10^{-4}	50	nil
Primary amine of chlorpromazine	10^{-4}	95	62

The components of the system were: sodium β-hydroxybutyrate, 0·033 M; cytochrome-*c* 4×10^{-5} M; sodium inorganic orthophosphate buffer, pH 7·2, 0·033 M; sodium cyanide, 10^{-4} M; versene, 10^{-3} M; mitochondria equivalent to 0·004 mg N in 0·1 ml of water or 0·075 M sucrose were added to start the reaction. Final volume 3·0 ml and temperature 20°. When water-treated mitochondria were used, DPN 1 μmole was added to the system. The reaction was followed for 10 min by observing the increase in optical density at 550 mμ. Readings were made at 1 min intervals against blanks without substrate, using the Unicam SP500 spectrophotometer with glass cells of 1 cm light path. The control rate for the 0·075 M sucrose system was 11·3 μmoles cytochrome-*c* reduced per mg N per hr, and for the water system it was 34 μmoles cytochrome *c* reduced per mg N per hr. The percentage inhibitions quoted are the mean of six determinations, the error being ±10 per cent.

system (see Dawkins *et al.*[8]). In Table 3 results of experiments with several phenothiazines are shown. Two types of mitochondrial preparation were used. Mitochondria suspended in 0·075 M sucrose possess an intact phosphorylation system, while those suspended in water transport electrons without any accompanying phosphorylation. The results in the table show clearly that the inhibitions found with the phosphorylating system are not seen when the non-phosphorylating system is used. There are two exceptions to this, stelazine and the primary amine of chlorpromazine. These both produce 95 per cent inhibition at a concentration of 10^{-4} M in the phosphorylating system, and still show considerable inhibition in the water system. Once more there appears to be some correlation between inhibitory activity and tranquillizing ability. Thus chlorpromazine at 10^{-4} M produces 50 per cent inhibition of cytochrome-*c* reduction, while its sulphoxide only inhibits by 15 per cent at 2×10^{-4} M. Compazine at 10^{-4} M produces 60 per cent inhibition, whereas its sulphoxide has no effect at the same concentration. Diethazine at 4×10^{-4} M inhibits by 22 per cent while mepazine

at the same concentration inhibits by 80 per cent. However, not only is the primary amine effective in this test, but acetyl promazine, a powerful tranquillizer, is of relatively low activity.

3. *Effect on microsomal DPNH–cytochrome-c reductase*

Our results with chlorpromazine[8] indicated that the microsomal DPNH–cytochrome-*c* reductase was insensitive to the inhibitor. However, since stelazine and the primary amine of chlorpromazine inhibited the reduction of cytochrome-*c* by β-hydroxybutyrate (see Table 3) we investigated the activities of these compounds on the microsomal enzyme. Table 4 compares the effect of chlorpromazine, stelazine, the

Table 4. Effect of some phenothiazines on microsomal DPNH–cytochrome-*c* reductase

Compound	Concentration (M)	% Inhibition of cytochrome-*c* reduction
Chlorpromazine	2×10^{-4}	nil
Chlorpromazine	4×10^{-4}	50
Stelazine	10^{-4}	35
Primary amine of chlorpromazine	2×10^{-4}	50
Promethazine	10^{-3}	nil

All components and conditions were as in Table 3 with the exception that DPNH, 0·00067 M was the substrate and microsomes equivalent to 0·0002 mg N were used as source of enzyme, and were added in 0·1 ml of 0·25 M sucrose. The control rate was 530 μmoles cytochrome-*c* reduced per mg microsomal N per hr. The percentage inhibitions quoted are the mean of four determinations the error being ±10 per cent.

primary amine of chlorpromazine, and promethazine on the reduction of cytochrome-*c* by DPNH in the presence of microsomes. It will be observed that some inhibition is found with the first three compounds, but none with promethazine, even at 10^{-3} M, and that the microsomal enzyme is relatively insensitive to the drugs.

4. *Effects on oxidative phosphorylation*

Table 5 shows the effects of a variety of phenothiazines on the phosphorylation coupled to the oxidation of succinate. It will be seen that a variety of effects is obtained. Chlorpromazine at a concentration of 2×10^{-4} M reduces the P/O ratio by 50 per cent. Promethazine at the same concentration does the same, and at 4×10^{-4} M almost completely abolishes the phosphorylation. In fact there is no correlation between this effect and tranquillizing ability, or between this effect and those already considered in this paper. In Table 6 the same inhibitors were tested against the phosphorylation coupled to the oxidation of β-hydroxybutyrate. Again no correlation between inhibitions and tranquillizing ability is observed. In general the inhibitions with this system are less marked than on the succinate system.

5. *Localization of the effect on oxidative phosphorylation*

In a previous paper[7] we have shown that chlorpromazine has no effect on the efficiency of the phosphorylation coupled to the reduction of cytochrome-*c* by β-hydroxy-

butyrate or L-glutamate, even at concentrations which reduce electron transport by 50 per cent. However, it completely uncouples the phosphorylation associated with the oxidation of ferrocytochrome-*c*. This property is shared by numerous other phenothiazines (Table 7). There is no correlation between this action and tranquillizing ability. Not all phenothiazines show the same selectivity as chlorpromazine. Promethazine, for example, will uncouple both the phosphorylations associated with succinate oxidation.[11] It is of extreme interest to note that its only effect on the phosphorylation

TABLE 5. EFFECT OF PHENOTHIAZINES ON PHOSPHORYLATION COUPLED TO OXIDATION OF SUCCINATE

Experiment No.	Compound	Concentration (M)	O_2 uptake (μatm.)	P uptake (μmoles)	P/O	% Inhibition of P/O
1.	nil	—	12·5	21·0	1·7	nil
	Chlorpromazine	2×10^{-4}	15·0	12·1	0·8	53
	Promethazine	2×10^{-4}	12·4	10·5	0·8	53
	Promethazine	4×10^{-4}	11·6	2·3	0·2	82
2.	nil	—	7·9	12·1	1·6	nil
	Mepazine	$3·3 \times 10^{-4}$	4·3	nil	—	100
	Mepazine	10^{-4}	7·4	10·6	1·5	nil
	Diethazine	$3·3 \times 10^{-4}$	6·8	4·8	0·7	53
	Diethazine	10^{-4}	6·9	8·8	1·3	19
3.	nil	—	13·4	21·8	1·6	nil
	Promazine	2×10^{-4}	12·4	7·2	0·6	63
	Primary amine of chlorpromazine	$6·7 \times 10^{-5}$	13·0	8·0	0·6	63
	Acetylpromazine	2×10^{-4}	13·1	22·6	1·7	nil
	Stelazine	$6·7 \times 10^{-5}$	13·8	17·6	1·3	20
	Compazine	2×10^{-4}	13·4	18·1	1·35	16
	Compazine sulphoxide	2×10^{-4}	13·8	23·8	1·73	nil
	Chlorpromazine sulphoxide	$6·7 \times 10^{-4}$	13·4	20·0	1·5	7

The components of the system were: ATP, 0·0017 M; $MgSO_4$, 0·0067 M; KCl, 0·025 M; cytochrome-*c* 2×10^{-5} M; sodium inorganic orthophosphate buffer, pH 7·2, 0·01 M; sodium succinate, 0·01 M; glucose 0·03 M; hexokinase, 0·05 ml. Mitochondria were pre-incubated in 0·25 M sucrose–0·02 M phosphate buffer, pH 7·2 for 10 min at 30° and added in 0·5 ml of this solution (equivalent to 0·3 mg N) Final volume 3·0 ml. Temperature 38°C. Duration of experiment 20 min, after 7 min thermal equilibration. Each experiment is representative of five similar determinations. Variations in the control figure for the P/O ratio were 1·6 ± 0·2.

coupled to β-hydroxybutyrate oxidation is to uncouple the terminal step (the phosphorylation coupled to the oxidation of ferrocytochrome-*c*). This must mean that promethazine uncouples the phosphorylation associated with succinate–cytochrome-*c* reductase, but not that associated with DPNH–cytochrome-*c* reductase.

6. *Other groups of tranquillizers*

Experiments were performed with tranquillizers of the diphenyl methane series and the substituted glycerols. None of these had effects comparable to the phenothiazines. Some of the diphenyl methane derivatives inhibited cytochrome oxidase at high concentrations, but there was no correlation with pharmacological activity. There were no effects on other parts of the respiratory chain. Reserpine could not be tested in these *in vitro* experiments because of its virtual insolubility.

Table 6. Effect of phenothiazine on phosphorylation coupled to oxidation of β-hydroxybutyrate

Experiment no.	Compound	Concentration (M)	O_2 uptake (μatm)	P uptake (μmoles)	P/O	% Inhibition of P/O
1.	nil	—	8·3	23·8	2·9	nil
	Chlorpromazine	2×10^{-4}	7·7	19·3	2·5	17
	Promethazine	2×10^{-4}	6·6	10·2	1·7	33
	Promethazine	4×10^{-4}	1·2	2·3	1·8	28
2.	nil	—	4·3	11·5	2·8	nil
	Diethazine	10^{-4}	3·7	6·0	1·6	43
	Mepazine	$3·3 \times 10^{-4}$	2·3	3·2	1·4	50
	Chlorpromazine sulphoxide	2×10^{-4}	4·5	12·2	2·7	nil
	Compazine	2×10^{-4}	5·0	11·5	2·3	17
	Compazine sulphoxide	2×10^{-4}	4·7	12·7	2·7	nil
3.	nil	—	5·3	12·1	2·3	nil
	Stelazine	2×10^{-5}	5·9	12·1	2·0	13
	Primary amine of chlorpromazine	2×10^{-5}	5·3	12·1	2·3	nil
	Promazine	10^{-4}	4·8	5·6	1·2	48
	Acetyl promazine	2×10^{-4}	5·6	9·3	1·6	31

All components and conditions were as for Table 5, except that sodium β-hydroxybutyrate, 0·01 M was the substrate. The mitochondria were pre-incubated in 0·075 M sucrose at 0°C for 30min before use and were added in 0·5 ml of this suspension (equivalent to 1·0 mg N) Duration of experiment 15 min after 7 min thermal equilibration. Each experiment is representative of five similar determinations. The variation in the control figure for the P/O ratio was 2·6 ± 0·3.

Table 7. Effect of some phenothiazines on phosphorylation coupled to oxidation of reduced cytochrome-*c*

Experiment no.	Compound	Concentration (M)	O_2 uptake (μatm)	P uptake (μmoles)	P/O	% Inhibition of P/O
1.	nil	—	10·9	6·5	0·6	nil
	Chlorpromazine	10^{-4}	9·0	1·6	0·18	70
	Diethazine	2×10^{-4}	9·5	1·2	0·13	78
	Mepazine	2×10^{-4}	10·1	3·6	0·32	47
	Promethazine	2×10^{-4}	9·3	1·6	0·17	73
2.	nil	—	12·4	6·4	0·52	nil
	Chlorpromazine	2×10^{-4}	11·0	nil	—	100
	Chlorpromazine sulphoxide	2×10^{-4}	11·2	5·3	0·48	7

All components as described in Table 5, except that the substrate was ascorbic acid, neutralised to pH 7·2 with NaOH just before use, 0·01 M. Mitochondria were pre-incubated in 0·075 M sucrose for 15 min at 0°C before use, and were added in 0·5 ml of this suspension (equivalent to 2·0 mg N). Temperature 20°C. Duration of experiment 60 min after 7 min thermal equilibration. Each experiment is representative of five similar determinations. The variation in the control figure for P/O ratio is 0·5 ± 0·1.

DISCUSSION

Mechanism of action

In this paper we have made no mention of the possible mechanism of action of these compounds. We have been studying this problem, and the interested reader can refer

to our other publications.[6–8,11] It is to be remembered that the phenothiazines have two points of action on the respiratory chain *in vitro*. The first is the inhibition of cytochrome oxidase, which is apparently competitive in nature, and which is reversed by increasing cytochrome-*c* concentration. The second is the inhibition of DPNH–cytochrome-*c* reductase. For the inhibition of cytochrome oxidase, we are unable to offer any explanation. The effect of DPNH–cytochrome-*c* reductase, we believe, is due to action not on the respiratory enzymes themselves but on the coupled phosphorylation system, though with some of the compounds tested some direct action may be observed.

The fact that some of the inhibitors affect uncoupled systems or non-phosphorylating DPNH–cytochrome-*c* reductases (e.g. the microsomal enzyme) is of interest. It suggests that the primary effect of the phenothiazines centres round the flavoproteins.

Correlation of pharmacological activity and biochemical effect

We had hoped that our enzymic tests would reveal some degree of correlation between biochemical activity and tranquillizing ability. The first difficulty in such a task is the absence of a generally agreed definition of a tranquillizer. The second is that the drugs may be metabolized or excreted in the whole animal, so that they reveal either more or less activity in isolated systems than is expected from animal tests. For example, in the present work we find that the primary amine of chlorpromazine shows high biochemical activity but little effect on the whole animal. Conversely, acetylpromazine is a potent tranquillizer which shows little biochemical activity. However, there is some correlation between inhibition of DPNH–cytochrome-*c* reductase and tranquillizing ability.

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The question now arises whether the biochemical and pharmacological actions are directly related. It is of interest that amylobarbitone has a very similar action on electron transport to the phenothiazines; that is, it inhibits DPNH–cytochrome-*c* reductase in phosphorylating systems, but has no effect when the phosphorylating process is disrupted.[12] The concentrations required for this effect are high, about 2×10^{-3} M, which is some ten times greater than the concentration of chlorpromazine giving a similar effect. Even a perfect correlation between inhibition of electron transport and tranquillizing ability need not necessarily mean that a direct relationship exists. One obvious discrepancy is the concentration at which biochemical activity is observed. For example, chlorpromazine at a concentration of 10^{-4} M, if evenly distributed throughout all the tissues of a 70 kg man represents a dose of 2·5 g of the drug present. Even if restricted to the circulating blood, an amount of some 250 mg is required. It could be postulated that even distribution never occurs, and that concentration may occur in some tissue or tissues. In this connexion Lindan *et al.*[4] suggest that chlorpromazine combines with tissue proteins and thereafter diffuses into the cells to bring about metabolic inhibitions. Another possibility is that some sensitive site exists, and for this the central nervous system is an obvious choice. Though no experiments with brain are reported in this paper, we have carried out experiments with brain mitochondria. These, in common with the experiments of Lindan *et al.* already quoted, reveal no special sensitivity to the drugs.

Finally, it is clear that respiratory chain inhibition need not be the basis for tranquillizing action. The tranquillizers in the diphenylmethane and substituted glycerol

series are very poor inhibitors of the cellular respiratory mechanism, even at very high concentrations.

SUMMARY

(1) The action of several phenothiazine derivatives was tested on the respiratory chain of liver mitochondria.

(2) Inhibition of cytochrome oxidase and DPNH–cytochrome-*c* reductase was found to be a characteristic of these compounds. The inhibition of cytochrome oxidase is reversed by increasing the concentration of cytochrome-*c*. The inhibition of DPNH–cytochrome-*c* reductase occurs only in phosphorylating systems.

(3) There is some correlation between inhibition of DPNH–cytochrome-*c* reductase and tranquillizing ability, but several anomalous results have been observed.

(4) Several of the phenothiazines tested exhibit specific inhibitory effects on the coupled phosphorylating reactions. However, the inhibitory action against oxidative phosphorylation is not a prominent feature of the biochemical action of these compounds.

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THE POISONING OF *MAIA SQUINADO* (HERBST) BY CERTAIN COMPOUNDS OF MERCURY

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Abstract—When *Maia* is poisoned by immersion in sea-water containing added $HgCl_2$, mercury concentrates at the gills and at various sites within the animal. Eventually the concentration of mercury in the blood rises above that in the external medium; the concentration in the antennary glands above that in the blood; and the animals excrete small but increasing amounts of the poison in the urine. A high proportion (95 per cent) of the mercury present in the blood is attached to protein, and the concentration of mercury in the blood remains fairly constant for several weeks after poisoned animals are returned to the sea. A similar pattern of mercury distribution is obtained when $HgCl_2$ is administered to the animals by injection, and there is evidence that animals so treated excrete small amounts of the poison through the gills. When *Maia* is poisoned with n-$C_5H_{11}HgCl$, mercury again concentrates at the gills and in various internal organs; but the amount detected in the blood is very small and none is found in the urine.

Amino acids present in the blood of normal *Maia* include alanine, α-aminobutyric acid, arginine, aspartic and glutamic acids, glutamine, glycine, *iso*leucine, leucine, lysine, proline, serine, taurine, threonine and valine. With the exception of taurine, all these amino acids are also present in the urine. In both these body fluids glycine, alanine, glutamic acid, proline and arginine preponderate; the other amino acids are present only in trace amounts. All fifteen amino acids, apparently in the same relative amounts, are also present in the blood of animals poisoned either with $HgCl_2$ or with n-$C_5H_{11}HgCl$; and, with the exception of taurine, in the urines of these animals. However, when the animals are immersed for progressively longer times in sea-water containing $HgCl_2$, or are injected with increasing amounts of this poison, the total quantity of amino-N in the urine rises, although that in the blood does not significantly change. There is, therefore, an increase in the urine: blood ratio of amino-N. This ratio also increases when the animals are immersed in sea-water containing n-$C_5H_{11}HgCl$, but in this case the level of amino-N in the blood rises as well as that in the urine. Animals poisoned with $HgCl_2$ and then returned to the sea still show an abnormally high urine: blood ratio of amino-N when examined several weeks later.

The levels of total sulphate in the urine and blood of the *Maia* used in this work were in the ratio 1·5 : 1. This ratio does not significantly change when the animals are poisoned with $HgCl_2$ under conditions that cause a marked increase in the excretion of amino-N.

The possible interference of mercury with mechanisms concerned with the metabolism of copper in *Maia* has been discussed, with especial reference to the influence of mercury on the functioning of the antennary glands.

INTRODUCTION

THE present work is a continuation of an earlier study using the prawn *Leander serratus* (Pennant) in which it was found that animals poisoned with $HgCl_2$ or with n-amylmercuric chloride (n-$C_5H_{11}HgCl$) concentrate mercury at the gills and in several

internal organs, notably the antennary glands.[1] These findings indicate that one of the ways in which the poisons might act on crustaceans is by interference with excretory processes, and this aspect of mercury poisoning has now been studied using the marine crab *Maia squinado* (Herbst).

Data on the use of clearance tests for assessing the efficiency of "kidney" function in arthropods are few and uncertain. Some work has been done on inulin clearance by the lobster *Homarus americanus*[2] and by the crayfish *Cambarus clarkii*.[3] However, the results are difficult to interpret. Thus, animals of the latter species are able to secrete inulin. Furthermore, no clearance tests of any kind appear to have been made with crabs. By contrast, however, several observations have been made on the effects of various external factors on nitrogen excretion by marine crustaceans,[4,5] and there is a good deal of information available on the levels of various organic and inorganic constituents in the blood and urine of *Maia*. Thus, it is known that the animal excretes a urine with a lower concentration of amino-N[6] and a higher concentration of sulphate[7] than that of the blood. Accordingly, it seemed likely that evidence of impaired renal function in *Maia* treated with mercury might be obtained by examining the levels of amino-N and sulphate in the blood and urine of poisoned animals, and this was the method used in the present investigation.

MATERIALS AND METHODS

Animals. Each of the crabs used in this study was a large male *Maia* that weighed approximately 1 kg. After the animals had been collected from the sea, they were brought into the laboratory and kept without food under circulating sea-water for 2 days before they were used in any experiments. Because part of the work was concerned with estimates of amino-N in the bloods and urines of the test animals, it was necessary to avoid spurious effects caused by moulting. Accordingly, no experiments were carried out during July and August, the moulting season for *Maia* at Plymouth.[8]

Poisons. Mercuric oxide that contained ^{203}Hg was supplied by the AERE, Harwell, and ^{203}Hg-labelled *n*-amylmercuric chloride (n-$C_5H_{11}HgCl$) was purchased from the Radiochemical Centre, Amersham. A non-radioactive sample of n-$C_5H_{11}HgCl$ was obtained from Lunevale Products Ltd.

Mercury analyses

Each of four pairs of animals was placed in a glass tank and covered with 10 l. of sea-water (obtained from Hydrographical Station E1) to which enough poison had been added to give a final concentration of either 10 mg Hg/l. (as $HgCl_2$) or 0·25 mg Hg/l. (as n-$C_5H_{11}HgCl$). The toxic media were aerated continuously, and after measured times, a pair of animals was withdrawn from the appropriate solution, rinsed quickly with fresh sea-water and analysed for mercury as follows.

Urine. Each operculum was gently opened with forceps and a polythene tube of thin bore was inserted into the orifice of the excretory canal.[9] This operation caused the animals to excrete urine at once and the clear, colourless liquid was collected by means of a syringe connected to the polythene tube. The urine from each of the two animals was pooled and samples (0·2 ml) were placed on planchettes and mixed with equal amounts of a 1 per cent aqueous solution of sodium sulphide. The mixtures were then slowly evaporated to dryness and examined for radioactivity. The correction necess-

ary for self-absorption in these samples was estimated by comparing the radioactivities of samples of solutions of blood, urine and distilled water to which the same quantity of ^{203}Hg had been added. The corrections so determined, which were applied in all subsequent calculations, were small, being only 5 per cent in the case of the blood and 3 per cent in the case of the urine. It was not necessary to apply a correction for self-absorption induced by the addition of Na_2S to the samples because this procedure was employed in the preparation of samples of stock solutions used daily as controls.

Blood. The walking-legs of each animal were cut off and its blood was drained through a muslin cloth into a beaker. Samples (0·2 ml) of the pooled blood from both animals were then treated in the same way as those of the urine.

Gills and exoskeleton. A whole gill was dissected from each animal and several of the lamellae were weighed, placed on planchettes, then warmed gently to dryness and examined for radioactivity. Several parts of the exoskeleton other than the gills were also analysed but only small amounts of mercury were detected and the results have not been included.

Fore-gut. Samples were cut from the walls of the oesophagus and cardiac chamber of the proventriculus, and their mercury contents were analysed in the manner just described.

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Internal organs. The heart, antennary glands and ventral nerve mass were excised whole; a sample (10 g) of the digestive gland ("hepatopancreas") was severed from that portion which lay above the anterior region of the branchial chamber; muscles were dissected from the region lateral to the ventral nerve mass. As soon as they had been dissected, samples of the same organ taken from each of the two crabs were mixed, drained free from body fluid with filter paper, and comminuted with a glass pestel and mortar. Each of several samples of the paste so formed (*c.* 50 mg) was then weighed and spread evenly on planchettes which were subsequently warmed gently to dryness and tested for radioactivity. It was found that if the total weight of fresh tissue on any one planchette did not exceed 10 mg, errors caused by self-absorption were negligible, even when the samples examined were those of densely packed tissue cut from the digestive gland and muscles.

Studies using paper chromatography

Experiments were carried out in order to identify the amino acids present in the bloods and urines of the experimental animals. This was done partly because it was thought advisable to know the composition of these mixtures of amino acids before deciding on a method of determining their total quantity; and partly because it was considered worth while to see if there were any marked differences between the qualitative patterns of the amino acids present in extracts of urines and bloods of poisoned and unpoisoned *Maia*.

Preparation of extracts. After their bladders had been emptied of urine, and their opercula had been sealed with dental cement, five animals were immersed in sea-water that contained 10 mg Hg/l. as $HgCl_2$; five more in sea-water that contained 0·25 mg Hg/l. as n-$C_5H_{11}HgCl$, and a further five in sea-water to which no additions had been made. After 7 hr, the animals were removed from these media, and the bloods and urines of each batch of five were collected in the usual way and pooled. Amino

acids were separated from these pooled bloods and urines by means of the procedure described by Kermack *et al.*[10] for experiments with *Homarus vulgaris*, and then examined on paper chromatograms using standard methods.[11, 12]

Estimations of amino-N

Before the experiments were begun the bladders of the test animals were emptied of urine and opercula were sealed with dental cement. Batches of five animals were then immersed for 3·5, 7, 12 and 19 hr in sea-water containing 10 mg Hg/l. as $HgCl_2$, and four similar batches were immersed for identical times in sea-water containing 0·25 mg Hg/l. as n-$C_5H_{11}HgCl$. As controls, further batches of animals were immersed in sea-water to which no additions had been made. At the end of each experimental period, the urines and bloods from all five animals in the appropriate batch were collected in the usual way and pooled. Amino acids were then separated from these body fluids in the usual way. Each of the final residues was dissolved in distilled water (10 ml), the solution so formed was adjusted to pH 9·5 with 1 N NaOH and its content of amino-N was then estimated by the method of Albanese and Irby[13].

Estimations of total sulphate

Urines and bloods were collected from poisoned and control animals and diluted 1 : 5 with distilled water. A sample (5 ml) of each diluted solution was mixed with an equal volume of 5 N HCl and evaporated to dryness on a boiling-water bath (20 min). The residue was then triturated with distilled water (6 ml), the suspension was filtered and the sulphate present in the filtrate was estimated by means of the benzidine-precipitation procedure.[14] The benzidine sulphate precipitates were filtered off and washed using an apparatus of the type described by Henriques *et al.*[15] and they were then titrated with 0·02 N NaOH using phenol red as indicator.

RESULTS

Distribution of mercury

The data in Table 1 describe the accumulation of mercury by various tissues and body fluids of *Maia* poisoned with $HgCl_2$. As large quantities of the poison were found in the blood and as the various tissues examined were not entirely freed from blood before they were analysed for mercury, the results in Table 1 may only be regarded as semiquantitative. Nevertheless the data adquately demonstrate that considerable quantities of the poison are taken up by the gills, oesophagus, proventriculus and antennary glands, for all these tissues eventually accumulated a far higher concentration of mercury than that found in the blood.

It is interesting to note that the quantity of mercury present in the blood increased during the experiment until its concentration was considerably greater than that of the external medium. Of further interest was the finding that the animals were able to excrete small quantities of the poison, traces of which were consistently detected in the urine.

The radioactivity present in the surrounding medium was measured at the beginning and end of each experiment so that the quantity of poison which had been accumulated by each pair of animals could be estimated. The results, expressed as mg Hg/kg body weight, are included in Table 1.

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It was important that none of the test animals should die during the course of these experiments, because the rapid rate of autolysis which follows death in *Maia* would have seriously influenced the results of complementary studies in which estimations were made of the levels of amino-N in the bloods and urines of the animals. Accordingly, the experiments with isotopes were not continued for more than 19 hr because it was found that when the animals were immersed in the toxic medium for periods slightly longer than this, they died.

TABLE 1. DISTRIBUTION OF MERCURY IN CRABS IMMERSED IN TOXIC SOLUTIONS OF $HgCl_2$ AND n-$C_5H_{11}HgCl$

Part of animal	Concentrations of Hg (μg/g fresh wt. of tissue or body fluid) after various times of immersion (hr)				
	Expt. with $HgCl_2$				Expt. with n-$C_5H_{11}HgCl$
	(3·5hr)	(7·0 hr)	(12 hr)	(19 hr)	(19 hr)
Gill lamellae	43–47	50–54	170–195	360–396	212–250
Oesophagus	1·4–1·7	7·8–8·2	29–33	120–130	18–26
Proventriculus	0·2–0·4	1·4–1·9	4·3–5·5	100–112	3·0–5·6
Antennary gland	0·3–0·5	2·2–2·3	18–21	53–61	25–30
Digestive gland	0–0·1	0·4–0·7	3·0–3·3	26–29	8·9–9·0
Blood	1·2–1·3	7·4–7·5	11–12	25–26	1·2–1·9
Ventral nerve mass	0·4–0·9	1·2–1·3	4·0–5·0	18–19	5·8–12
Heart	0·2–0·3	1·3–1·6	3·9–4·7	15–16	8·2–17
Abdominal muscle	0	0·4–0·7	0·8–0·9	2·1–2·3	0·2–1·2
Urine	0	0	0·7–0·8	1·6–1·8	0
Total quantity of Hg accumulated by animal (mg/kg body wt)	7·8	34·5	48·9	59·0	

Pairs of animals immersed in sea-water containing 10 mg Hg/l. as $HgCl_2$ and 0·25 mg Hg/l. as n-$C_5H_{11}HgCl$. Figures show the range of five determinations made on a sample of pooled tissue from two animals.

Mercury attached to protein

It seemed possible that the concentration of mercury found in the blood of *Maia* rose markedly above that of the surrounding medium because large amounts of the metal became attached to the blood protein. This possibility was examined in the following way. Blood from animals that had been immersed for 19 hr in sea-water which contained 10 mg Hg/l. (as ^{203}Hg-labelled $HgCl_2$) was treated with one-fifth of its volume of a 4 per cent aqueous solution of trichloracetic acid. The copious white precipitate that formed was removed by filtration and, together with the filtrate, was examined for radioactivity. The results of this experiment showed that 95 per cent of the total radioactivity present in the blood could be detected in the protein fraction, and only 5 per cent in the non-protein fraction. The values obtained when the urine was examined were 68 and 31, respectively. The experiment was repeated using 95 per cent ethanol as the protein-precipitant and a similar result was found.

Experiments with n-*amylmercuric chloride*

It seemed possible that as n-$C_5H_{11}HgCl$ is much more lipoid-soluble than $HgCl_2$, but does not react to the same extent with proteins, it might show a pattern of dis-

tribution in *Maia* different from that observed with $HgCl_2$. This possibility was tested by carrying out experiments with n-$C_5H_{11}HgCl$ that were similar to those done with $HgCl_2$.

The results of these experiments are also included in Table 1 from which it will be seen that a concentration of n-$C_5H_{11}HgCl$ equivalent to 0·25 mg Hg/l. in the toxic medium caused certain internal organs of the test animals, notably the antennary glands and the ventral nerve masses, to accumulate more than half the corresponding concentrations found in these tissues when the toxic medium contained 10 mg Hg/l. as $HgCl_2$. This finding, therefore, demonstrated that mercury in the form of the highly lipoid-soluble compound n-$C_5H_{11}HgCl$ concentrated very rapidly at sites within the test animal.

Of further interest was the finding that in experiments with n-$C_5H_{11}HgCl$ the relative concentrations of mercury located in various parts of the test animals were markedly different from that observed in experiments with $HgCl_2$. Thus, with the exception of the abdominal muscles, all the internal tissues of animals that had been poisoned with n-$C_5H_{11}HgCl$ contained mercury greatly in excess of the concentration present in the blood. By contrast, among the internal organs of the animals poisoned with $HgCl_2$, only the antennary glands possessed a concentration of mercury higher than that present in the blood, and the extent of the accumulation was much less than that observed in the corresponding experiments with n-$C_5H_{11}HgCl$. Similarly, examination of various external sites of attachment showed that the concentrations of mercury on the gills and oesophagus of the test animals, relative to the amount present in the blood, were far greater when n-$C_5H_{11}HgCl$ was used as the poison than when the crabs were treated with $HgCl_2$.

Uptake of mercury by the gills

Experiments described earlier had shown that when *Maia* is immersed in solutions of $HgCl_2$, very large quantities of the poison are accumulated at the gills. It seemed possible that the poison may not have concentrated at this site solely by accumulation from the external medium: some of it may have represented mercury that the animals were trying to excrete through the gills into the surrounding sea-water. To examine this possibility, experiments were carried out in which $HgCl_2$ was injected into the animals which were then dissected after various times and analysed for mercury in the usual way. Ten animals were used and each received a dose of 5 mg Hg/kg body weight as a solution of $HgCl_2$ in *Maia* Ringer,[16] the poison being injected directly into the sternal sinus by inserting the needle ventrally through the arthrodial membrane between the ventral body wall and the coxopodite of the cheliped. Immediately after the animals were injected, urine was removed from their bladders in the usual way and their opercula were sealed with dental cement. The animals were then immersed in sea-water until required for analysis. During the early stages of the experiment (0–19 hr) analyses were made of the radioactivity present in the external medium, and the values obtained were 0·3, 0·8, 5 and 7 μg Hg/l. at 3·5, 7, 12 and 19 hr, respectively, after the animals had been injected with the poison. These findings showed that traces of mercury were excreted by *Maia* into the surrounding medium, and as the animals did not produce faeces and as the opercula were sealed throughout the experimental period, it seems reasonable to suppose that this mercury was excreted via the gills. Further evidence consistent with this view is summarized in Table 2.

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The experiments showed that after *Maia* is injected with $HgCl_2$, mercury slowly spreads into its tissues and concentrates to a very large extent at the gills and in the antennary glands. Maximal concentrations were observed at these sites after 19 hr, and the amounts present after this time in the blood, ventral nerve mass, heart and digestive gland approximated to the quantities of mercury found in these tissues in earlier experiments in which the animals were immersed for 3·5–7 hr in sea-water containing 10 mg Hg/l. as $HgCl_2$ (see Table 1). By contrast, the amounts of mercury detected in the oesophagus and proventriculus of the injected animals were much less than those observed in samples taken from the corresponding tissues of animals

TABLE 2. DISTRIBUTION OF MERCURY IN CRABS INJECTED WITH $HgCl_2$

Part of animal	Concentration of Hg. (μg/g fresh wt. of tissue or body fluid) at various times after injection with $HgCl_2$				
	(1 hr)	(2 hr)	(19 hr)	(2 weeks)	(4 weeks)
Gill lamellae	1·5–1·7	1·7–2·3	57–106	16–18	8·4–9·3
Oesophagus	0·1–0·5	0·1–0·4	0·1–0·4	0·2–0·6	0·3–0·4
Digestive gland	0·2–0·4	0·4–0·5	0·5–0·9	0·1–0·8	0·7–1·0
Heart	0·2–0·3	0·5–0·6	2·1–2·3	1·7–1·8	1·1–1·5
Abdominal muscle	0·2–0·3	0·5–0·6	1·0–1·3	0·2–0·8	0·5–0·8
Ventral nerve mass	0·1–0·3	0·4–0·5	1·2–3·1	1·9–2·1	0·7–1·3
Antennary gland	0·5–0·8	0·9–1·0	4·5–4·7	2·1–3·0	1·7–2·3
Blood	1·9–2·0	2·4–2·6	3·2–3·4	2·9–3·0	2·8–2·9
Urine	0·1–0·2	0·5–0·6	1·8–2·0	0·3–0·4	0·5–0·7

Each animal received 5 mg Hg/kg body wt as a solution in *Maia* Ringer. Figures show the range of five determinations made on a sample of pooled tissue from two animals.

immersed in a sea-water solution of $HgCl_2$. Further comparison of the relevant data in Tables 1 and 2 shows that the quantities of mercury which concentrated in the internal organs of animals that had been injected with 5 mg Hg/kg body weight approximated to the concentrations observed in the same tissues when the animals had taken up roughly 20 mg Hg/kg body weight from the surrounding sea-water (7·8 mg after 3·5 hr and 34·5 mg after 7 hr, Table 1). It seems therefore, that only about 25 per cent of the mercury accumulated by *Maia* from an external solution containing 10 mg Hg/l. as $HgCl_2$ actually reaches the internal organs: the other 75 per cent must presumably be attached to the animal's surfaces.

As the animals did not die in this experiment, it was continued for longer than 19 hr. However, the *Maia* could not be kept in the laboratory under sea-water that was also circulating through a public aquarium because they contained radioactive mercury. Accordingly, after their opercula had been freed from dental cement, the test animals were confined in a steel cage, supplied with a diet of dead squid and submerged in Plymouth Sound. A pair of animals was brought into the laboratory for analysis after 2 weeks, and a further pair after 4 weeks. The results of these analyses (included in Table 2) showed that many of the tissues of these *Maia* lost considerable quantities of mercury during the 4 weeks' immersion in the sea. However, an important exception to this was the blood, which retained a relatively high and fairly constant concentration of mercury throughout the experimental period.

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Paper chromatrographic studies

The results obtained using paper chromatography indicated that the amino-acid fraction of every mixture examined contained large quantities of alanine, glycine, arginine, proline and glumatic acid, together with trace amounts of α-aminobutyric and aspartic acids, glutamine, leucine/*iso*leucine, lysine, serine, threonine and valine. Further experiments were carried out in which all these amino acids were added to desalted extracts of blood and urine which were then examined by means of two-dimensional paper chromatograms. When this was done it was found that the pattern of spots obtained was identical with that observed when a chromatogram was prepared using a corresponding extract to which no additions of amino acids had been made. The result of this "fingerprinting" experiment therefore provided further evidence in support of the view that these extracts only contained the amino acids listed above.

Although no attempt was made to carry out accurate estimations of the relative quantities of amino acids present in the bloods and urines of poisoned and control animals, examination by eye of the sizes of spots on appropriate chromatograms indicated that the relative amounts of the amino acids in the blood and in the urine of normal *Maia* were similar; also that there was no marked difference between the relative amounts of the amino acids present in the blood and urine of the animals poisoned either with $HgCl_2$ or with n-$C_5H_{11}HgCl$. However, during these preliminary experiments it became obvious that there were considerable differences between the total concentrations of amino-N in the urines of the poisoned and control animals, and a more detailed study of these differences was therefore made.

Table 3. Influence of mercury on urine : blood ratio of amino-N

Time (hr)	Urine/blood amino-N	
	Expt. with $HgCl_2$	Expt. with n-$C_5H_{11}HgCl$
0	0·15	0·19
3·5	0·29	0·32
7·0	0·37	0·50
12	0·43	0·41
19	0·44	0·54
Control	0·13	0·16

Animals immersed in sea-water containing 5 mg Hg/l. as $HgCl_2$ and 0·25 mg Hg/l as n-$C_5H_{11}HgCl$. Five animals used for each determination. Opercula of first five batches sealed with dental cement. Opercula of sixth batch ("control") unsealed.

Quantitative studies

When *Maia* was immersed for progressively longer times in sea-water containing $HgCl_2$, the level of amino-N in the urine rose (0·48–1·26 mg amino-N/100 ml in 19 hr). However, this increase was not attended by a corresponding change in the level of amino-N in the blood: the latter value remained fairly constant at 2·81 mg amino N/l. Because of this, throughout the experiment, the ratio urine : blood amino-N increased steadily until, at the end of 19 hr, it had attained approximately three times its normal value (see Table 3).

When an identical study was made with n-$C_5H_{11}HgCl$, similar results were obtained until the experimental period was extended beyond 7 hr. After this time the level of amino-N in the blood showed a marked increase (4·67 mg amino-N/100 ml at 12 hr) that became greater still after 19 hr. As in the experiments with $HgCl_2$, urinary levels of amino-N increased throughout the experiment (0·58–2·72 mg amino-N/100 ml in 19 hr) but, because of the rise in the blood levels, the ratio urine : blood amino-N in the experiments with n-$C_5H_{11}HgCl$ did not show a steady increase after 7 hr.

It has been shown that amputation of the walking-legs in fasting *Carcinides maenas* (Pennant) causes a large increase to occur in the output of urinary nitrogen during the first day after the operation.[4] It is not known if the same thing happens in the case of *Maia*, but the use of control animals in the present series of investigations showed conclusively that the observed increases in urinary amino-N were caused by mercury poisoning and not by amputation of the walking-legs for the purposes of collecting blood. However, as a further safeguard, it was considered necessary to include among the controls a further batch of *Maia* that had not undergone the operation necessary for removing urine from the bladders and whose opercula remained unsealed throughout the experimental period. The data obtained with these *Maia* are included in Table 3 and show that analyses using both treated and untreated control animals gave similar results. Thus, the effect of this treatment on the levels of amino-N in the blood and urine were negligible.

Injection experiments. A further series of experiments was made in which the bloods and urines of *Maia* were examined 19 hr after the animals had been injected with different quantities of $HgCl_2$ dissolved in *Maia* Ringer. The results of these experiments are shown in Table 4 and demonstrate that when the size of the dose administered was increased from 2–10 mg/kg body weight the ratio urine : blood amino-N rose steadily. It was found that animals which had received 10 mg Hg/kg body weight showed a marked increase in the level of amino-N in their blood as well as their urine. However, the ratio urine : blood amino-N was still higher for these animals than for those used as controls.

TABLE 4. INFLUENCE OF INJECTED $HgCl_2$ ON URINE : BLOOD RATIO OF AMINO-N

Size of dose injected (mg Hg/kg body wt.)	Time after injection	Urine/blood amino–N
0	0 hr	0·20
2	19 hr	0·27
5	19 hr	0·32
10	19 hr	0·36
5	4 weeks	0·27

Four animals used for each experiment.

Persistence of toxic effect. Earlier experiments with tracer isotopes had shown that 1 month after they had been injected with mercury, *Maia* still excreted traces of the poison in the urine (see Table 2). An experiment was therefore carried out in which analyses were made of the levels of amino-N in the blood and urine of twenty animals 1 month after each had received 5 mg Hg/kg body weight as $HgCl_2$ by injection and

had then been returned to the sea. Sixteen of the animals survived and the analyses showed that after 4 weeks in the sea the ratio urine : blood amino-N was still higher for the poisoned (0·27) than for the control animals (0·20). A similar experiment was made in which the poison was administered not by injection but by immersing the animals for 3·5 hr in sea-water containing 10 mg Hg/l. as $HgCl_2$. Twenty animals were so treated and then returned to the sea. When they were examined 4 weeks later it was found that all had survived although the ratio urine : blood amino-N was still significantly high (0·31).

Influence of mercury on excretion of sulphate

It was found (see Table 5) that there was no significant change in the ratio of the levels of total sulphate in the bloods (average value, 336 mg SO_4^{2-}/100 ml) and urines (average value, 466 mg SO_4^{2-}/100 ml) in *Maia* poisoned by immersion for different times in sea-water containing 10 mg Hg/l. as $HgCl_2$, i.e. under conditions that gave rise to a marked increase in the quantity of amino-N in the urine (see Table 3).

Table 5. Influence of mercury on urine : blood ratio of SO_4^{2-}

Time (hr)	Urine/blood SO_4^{2-}
0	1·50
3·5	1·35
12	1·31
19	1·39
Control	1·42

Four animals used for each determination. Animals immersed in sea-water containing 5mg Hg/l. as $HgCl_2$. Opercula of first four batches of animals sealed with dental cement. Opercula of fifth batch ("control") unsealed.

DISCUSSION

Several studies have been made of the functions of the antennary glands in crustaceans, and results in general favour the view that these organs are able to carry out certain operations characteristic of the mammalian kidney. These include filtration, reabsorption and secretion.[17] No combined histological and physiological study seems to have been made using *Maia*, but it is likely that the formation of urine by this animal includes the following mechanisms. Proteins and amino acids are partially removed from the blood by filtration; quantities of the amino acids are reabsorbed into the blood from the filtrate against a concentration gradient and therefore by means of an active process; inorganic sulphate is secreted from the blood into the filtrate, possibly also against a concentration gradient (the SO_4^{2-} content of the urine is higher than that of the blood). In the present work it has been shown that the abnormally high levels of amino-N present in the urine of animals poisoned with mercury are not accompanied by significant changes in the amounts of urinary sulphate, and

these findings therefore imply that concentrations of mercury which influence either filtration or reabsorption have no effect on the process of secretion.

As the respiratory pigment in *Maia* is haemocyanin, this species would be expected to contain considerable quantities of copper. Analyses have been made by F. A. J. Armstrong of the amounts of copper present in the blood and urine of the animals used in the present work; and Zuckerkandl[18] has measured the levels of copper in the digestive gland of *Maia* at various stages in the intermoult cycle. These data are included in Table 6, which also shows the rates of assimilation of mercury in the blood, urine and digestive gland of *Maia*; and the rate of increase in the urine : blood ratio of amino-N. It will be seen from this table that at a time (7 hr) when the test animals were excreting more than twice the normal amount of amino-N, the concentrations of mercury in the blood, urine and digestive gland were considerably less than the corresponding quantities of copper in a normal animal. These findings stress the need to consider why an animal accustomed to the presence of large amounts of copper in its tissues and body fluids should be poisoned by much smaller quantities of mercury.

TABLE 6. TISSUE CONCENTRATIONS OF COPPER IN ANIMALS WITH AN INCREASED URINE : BLOOD RATIO OF AMINO-N INDUCED BY MERCURY POISONING

Time of immersion (hr)	Blood (mg/l.)	Urine (mg/l.)	Digestive gland (μg/g fresh wt.)	Urine/blood amino-N
	Concentrations of mercury			
3·5	1·27	0·017	0·10	0·29
7·0	7·45	0·057	0·53	0·37
12	11·6	0·76	3·1	0·43
19	25·1	1·64	27·5	0·44
	Concentrations of copper			
—	55·5	0·71	16–245	0·15

Animals immersed in sea-water containing 10 mg Hg/l. as $HgCl_2$.

It has been shown that the amount of urine formed daily by *Maia* is equivalent to 3 per cent of the body weight.[6] As the animals used in the present work weighed approximately 1 kg, and as the urine contained 0·71 mg Cu/l., it follows that they excreted about 20 μg Cu/day. If it be assumed that the animals took up only sufficient copper to replenish that which they excreted in the urine, then they assimilated approximately 20 μg Cu/day, presumably by extraction from the surrounding sea water which contained 21 μg Cu/l. However, in experiments with $HgCl_2$ it was found that the animals removed much larger quantities of mercury from the external medium (34·5 mg in 7 hr). Although further experiments showed that only some 25 per cent of the mercury was actually assimilated into internal tissues, even if it be assumed that all the copper taken up by the animals reached these sites, the inflow of mercury was still far greater than that of copper. As a considerable amount of the assimilated mercury was attached to blood proteins, and as smaller quantities of the poison were excreted in the urine, it seems that mercury became involved in the mechanisms normally concerned in the metabolism of copper; and thus it is possible that mercury

was toxic to *Maia*, even at a time when its final concentration in the animal was still significantly less than that of copper, because mechanisms involved in the slow metabolic turnover of copper had to deal with much larger amounts of incoming mercury and proved inadequate. For instance, the antennary glands, in addition to coping with their normal output of copper (20 μg/day), had also to excrete increasing amounts of mercury as the animals remained for progressively longer times in sea-water containing $HgCl_2$. Reference to Table 6 shows that after 19 hr in the toxic medium the urine of the poisoned animals contained 1·64 mg Hg/l., presumably as well as the normal concentration of copper (0·71 mg/l.). It would not be surprising therefore if, under these conditions, damage was done to the antennary glands, and the results of experiments discussed earlier have provided evidence consistent with this view.

There are, however, other possible ways in which mercury might influence mechanisms involved in the slow metabolic turnover of copper in *Maia*. These include partial displacement of copper from haemocyanin, or attachment to sites on the protein for which copper has no affinity. Both these changes might impair respiratory function and lead to a general disturbance of metabolism, and either could be induced by small amounts of mercury. There is also the possibility that the large quantities of mercury found in the gills of the test animals gave rise to toxic effects; for these organs, possibly involved in the assimilation of small amounts of copper, may have been damaged by having to deal with much larger concentrations of mercury present in the surrounding sea-water. There is also evidence that *Maia* may excrete some of the assimilated mercury through the gills into the external medium. Mercury accumulated by the gills in either of these two ways may cause impairment of respiratory exchanges and so lead to a disturbance of metabolism that results in an increased excretion of amino-N.

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THE REACTIVITY OF RADIOMIMETIC COMPOUNDS—III
CROSSLINKING OF NUCLEOPROTEIN*

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Abstract—The *in vitro* reaction of the nucleoprotein from herring sperm heads has been studied with representative members of the several classes of biologically active alkylating agents (nitrogen mustards, epoxides, ethyleneimines, esters of methanesulphonic acid). Treatment with bifunctional reagents results in crosslinking of the DNA component which can be isolated as a gel. The protein moiety does not take part in this crosslinking reaction which is confined to the joining together of different DNA molecules.

The relative crosslinking effectiveness varies, but in general follows the biological activity as assessed by the ability to inhibit the growth of tumours. Quantitative considerations suggest that the joining together of DNA molecules in the nucleoproteins of the cell nucleus provides an adequate mechanism for the much greater cytotoxic activity of bifunctional as opposed to monofunctional alkylating agents.

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A CHARACTERISTIC property of the radiomimetic alkylating agents (i.e. compounds having a nitrogen mustard, epoxide, ethyleneimine or methane sulphonic acid ester group) is that their cytoxic action, for example the inhibition of the growth of tumours is much more pronounced if the compounds carry more than one alkylating group per molecule.[1]† This led to the suggestion that these compounds functioned *in vivo* by joining two chromosome threads together[3] thereby giving rise to chromosome breaks in subsequent mitosis. We have carried out experiments *in vitro* to test this crosslinking hypothesis.[4] When solutions of deoxyribosenucleic acid (DNA) are treated with bifunctional radiomimetic alkylating agents, crosslinks are formed but most of these are intra-molecular and join together different groups within the same flexible molecule.[5, 6] The results reported in this paper deal with the effect on DNA when it is treated in the form of nucleoprotein so as to simulate more closely the situation in the cell. Preliminary results[5, 6] had shown that when nucleoprotamine from herring sperm was reacted with these alkylating agents, intermolecular crosslinking occurred. Essentially the same process has been observed with different nucleoproteins, but the most convenient material for detailed study were the sperm heads obtained from herrings. These can readily be isolated in a pure form.[7] They are made up almost entirely of DNA and protamine. Other constituents such as the nuclear membrane constitute less than 2 or 3 per cent of the total mass.

EXPERIMENTAL

Preparation of sperm heads. Herring testes were excised immediately after the fish had been caught, placed in a vacuum flask containing solid CO_2 and transported to the laboratory in London. The organ was minced in a cold room and plasmolysed by suspension in ice cold water. The sperm heads were separated by centrifugation and

*Part II published in *Biochem. Pharmacol.* **1**, 25 (1958).

†Out of the many hundred components tested only two exceptions were found to this rule. Both are monofunctional ethyleneimines which contained another group in the molecule that could take part in crosslinking[2].

washed several times in 0·01 M sodium citrate adjusted to pH 7. The sperm heads were stored at −70°C before use.

Alkylation. A suspension of sperm heads in 0·002 M sodium chloride was prepared which, for most of the experiments contained 1 per cent DNA. Since the sperm heads consist approximately of one-third DNA and two-thirds protamine, the total concentration of sperm heads suspended was 3 per cent. These suspensions are milky white and of low viscosity since the sperm heads do not swell in water and remain as dense spheres with a water content of about 20 per cent. The alkylating agent was added to these suspensions and reaction was allowed to take place at 37°C. During the reaction with the mustards and methanesulphonic acid esters acid is liberated as a result of hydrolysis of these compounds. With the epoxides and ethyleneimines alkali will be released when reaction occurs with the salts of acid groups such as the phosphate groups of DNA. To maintain the pH constant at pH 7 alkali or acid was added throughout the reaction automatically by using a Pye electrometric titration unit.

With the nitrogen mustards which react rapidly, the period of treatment was such that more than 87 per cent had either combined with the nucleoprotein or had been destroyed by hydrolysis with water. From the amount of acid liberated during the reaction an approximate estimate of the relative amounts of the two processes could be obtained. With the other alkylating agents there was no way by which the quantity combined with the sperm heads could be determined (see p. 141).

The amount of alkylating agent that had combined with the sperm heads was obtained directly for the aromatic nitrogen mustards since these have a strong absorption in the ultra-violet. At the end of the reaction an aliquot of the sperm head suspension in 0·002 M sodium chloride was centrifuged at 5000 *g* for 30 min which gave a perfectly clear supernatant in which the concentration of hydrolysed mustard was determined spectrophotometrically. Knowing the amount of mustard originally added the quantity bound by the sperm head could be calculated. Control experiments showed that the hydrolysed mustard is not absorbed by sperm heads so that the material bound must be combined by a chemical reaction.

Measurement of crosslinking. When sperm heads are treated with solutions of 2 M sodium chloride they swell instantly and eventually the gel formed disperses to give a highly viscous solution. To ensure that the gel dissolves, the sperm heads must be added to the concentrated salt solutions slowly and with constant shaking and the final concentration of DNA should be not more than 0·04 per cent and preferably 0·01 per cent. If these precautions are observed the nucleoprotein goes into true solution which is unaffected by spinning at 20,000 *g* for 2 hr.

Sperm heads treated with the bifunctional alkylating agents swell and then appear to disperse like the untreated, but on centrifuging at 20,000 *g* for 2 hr in a refrigerated centrifuge (5°C) a part of the DNA is spun out as a gel. The proportion of DNA which has been turned into gel was determined by measuring the phosphorus concentration of the supernatant solution before and after spinning using a colourimetric method.[8] The concentration of protamine in the 2 M sodium chloride solution was determined by the Sakaguchi reaction[9] before and after centrifguation and with none of the treatments was any difference observed proving that the gel is made up entirely of DNA.

Radiomimetic compounds. All of these were obtained in a pure form from members of the organic chemistry department of this Institute, by whom they were synthesized. The only exception being the nitrogen mustard HN2 and propylene oxide which were

TABLE 1. ALKYLATING AGENTS USED IN REACTION WITH NUCLEOPROTEINS

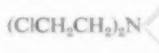
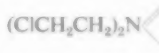
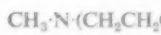
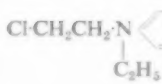
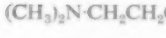
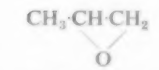
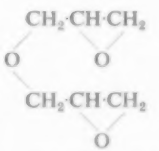
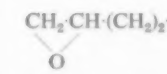
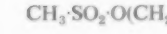
Name or code number	Formula	Reference to preparation
Bifunctional nitrogen mustards		
CB. 1045		*J. Chem. Soc.* 183 (1949,
CB.1348 (chlorambucil)		*J. Chem. Soc.* 2386 (1953)
CB.3039		*J. Chem. Soc.* 3835 (1955)
CB.3025 (melphalan)		*J. Chem. Soc.* 2409 (1955)
CB. 1385		*J. Chem. Soc.* 890 (1955)
CB. 1644		(Ross and Everett (1958) unpublished)
HN2		Bought from Boots Pure Drug Co.
Monofunctional nitrogen mustards		
CB. 1610		(Ross and Wilson (1957) unpublished)
CB. 3135		*J. Chem. Soc.* 90 (1959)
HN1		*J. Chem. Soc.* 513 (1947)
Other alkylating agents		
Propylene oxide		Redistilled from lab. suppliers
CB. 1161		*J. Chem. Soc.* 3131 (1950)
CB. 1170		*J. Chem. Soc.* 3131 (1950)
Myleran (CB. 2041)		*Biochem. Pharmacol.* **1**, 48 (1958)

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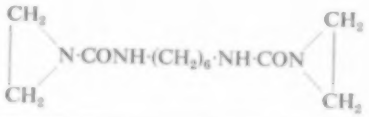
TABLE 1. ALKYLATING AGENTS USED IN REACTION WITH NUCLEOPROTEINS—*cont.*

Name or code number	Formula	Reference to preparation
Dimethyl myleran (CB. 2348)	$CH_3{\cdot}SO_2{\cdot}O{\cdot}CH(CH_3){\cdot}(CH_2)_3{\cdot}CH(CH_3){\cdot}O{\cdot}SO_2CH_3$	Hudson and Marshall, unpublished
CB. 1528	$CH_3{\cdot}CH_2{\cdot}O{\cdot}SO_2CH_3$	*Biochem. Pharmacol.* **1**, 48 (1958)
CB. 1263		*Liebig's Ann.* **556**, 210 (1950)

commercially available. The compounds are listed in Table 1 and will be referred to throughout the text by their code numbers.

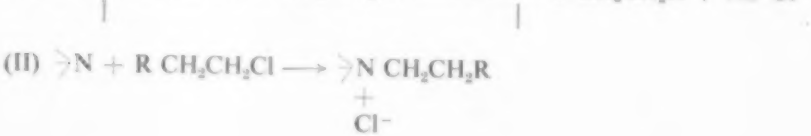
RESULTS

Reaction with mustards

The reaction with all the nitrogen mustards is similar though the nature of the group (M) to which the alkylating centre is attached (i.e. M = $N(CH_2CH_2Cl)_2$) determines the relative affinity (or competition factor)[4] for the substrate with which it is reacting. Any mustard that does not react is hydrolysed by the water in which the sperm heads are suspended. The higher the competition factor for nucleoprotein the greater is the proportion that combines. With the aromatic nitrogen mustards the reaction can readily be followed at pH 7 by measuring the amount of acid released, but this is not possible with the simpler aliphatic mustards such as HN2 since these are stronger bases and changes in the dissociation constant upon reaction modify the amount of acid bound as amine hydrochlorides.

The aromatic nitrogen mustard, CB. 3025, with its very high competition factor for nucleoprotein was used for detailed investigation of the nature of the reaction and the results described in the next section are largely confined to this substance, although there seems little doubt that the conclusions can be extended to other nitrogen mustards.

Site of reactions. Fig. 1 shows a typical experiment in which suspensions of sperm heads were treated with two different nitrogen mustards. The top curves show the amount of acid released by hydrolysis in 0·002 M salt only while the lower curves show the amount of acid formed in the presence of the sperm heads. The difference must be due to the esterification of an acid group (reaction (I) and the quaternization of a tertiary amino group (reaction (II)) since these are the only possible reactions in which a mustard does not give rise to release of acid.



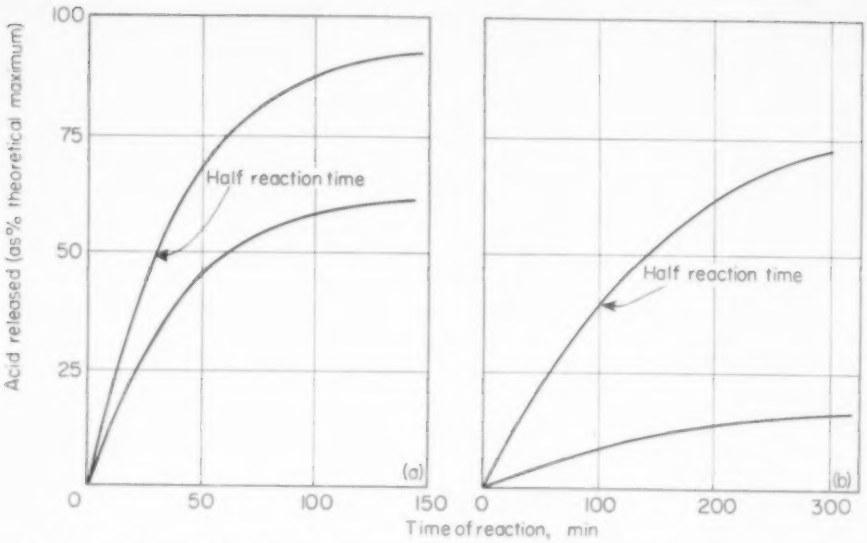


FIG. 1. Rate of acid formation during reaction of nitrogen mustards at 37° C.
(*a*) (*Top curve*) 0·105 per cent CB. 1348 in 10^{-3}M NaCl (reaction less than theoretical because of alkylation of carboxyl group of the mustard). (*Bottom curve*)—with 3 per cent sperm heads present.
(*b*) (*Top curve*)—0·08 per cent CB. 3025 in 10^{-3}M NaCal. (the maximum reaction corresponded to 88 per cent of theoretical probably because the compound as received contain some methanol of crystallization). (*Bottom curve*)—with 3 per cent sperm heads present.

The competition factor of CB. 3025 for sperm heads is high so that very little of it reacts with the water. With CB. 1348 an appreciable amount is lost by hydrolysis. Since the total amount of mustard combined with the sperm heads, determined spectrophotometrically, agrees within experimental error with the difference in the titration curves (see Table 2) it can be concluded that the reaction must have been entirely by

TABLE 2. REACTION OF HERRING SPERM HEADS WITH NITROGEN MUSTARDS

Conc. mustard (%)	Conc. nucleoprotein (%)	Mustard reacted with nucleoprotein (%)	
		By spectroscopic analysis of solution	From acid released during reaction
CB. 1348			
0·094	12	52	58
0·055	12	61	55
0·097	3	34	30
0·052	3	29	35
0·024	3	36	32
0·036	0·6	26	20
0·105	0·6	18	16
CB. 3025			
0·084	12	81	86
0·042	12	—	94
0·078	3	72	78
0·028	3	78	85

esterification (or possibly quaternization) and that there has been no detectable reaction with the amino (or imino) groups of the protamines or with the purine and pyrimidine residues of the nucleic acid (reaction (III)).

(III) $-NH_2 + R{\cdot}CH_2CH_2Cl \longrightarrow -NH{\cdot}CH_2CH_2R + HCl$

When very small amounts of this nitrogen mustard are used no acidity is produced showing that all the mustard must have been used up in reactions (I) and (II) and that none was lost by hydrolysis or by combination with amino groups as both these reactions give rise to acid.

The protein moiety of the sperm heads is made up entirely of the basic protamines which contain many amino groups, but only one anionic group per molecule, the terminal carboxyl group. In the absence of reaction with amino groups it is therefore most unlikely that an appreciable fraction of the mustard has combined with the protamine of the sperm head. This is in agreement with the finding that the gel produced by treatment with bifunctional alkylating agents contains only DNA and no protamine. The much lower competition factor of the mustard for proteins than for the DNA component of nucleoprotein is also shown by the fact that the presence of large quantities of serum albumin have little influence on the amount of DNA crosslinked by CB. 3025 (see Table 3) even though serum albumin is alkylated by the mustard in

TABLE 3. EFFECT OF PRESENCE OF PROTEIN ON CROSSLINKING OF SPERM HEADS* BY MUSTARDS

Conc. of serum albumin (%)	Conc. of mustard (%)	DNA present as gel (%)
CB. 3025		
0	0·021	68
1	0·021	63
5	0·021	58
CB. 1348		
0	0·124	61
5	0·124	11
0	0·050	43
5	0·050	0

* A suspension containing 3 per cent nucleoprotein was used.

the absence of nucleoprotein.[10] Protein, however, actively competes with the nucleoprotein for CB. 1348 and with a mustard which has a high competition factor for proteins[10] the extent of crosslinking of nucleoproteins is greatly reduced in the presence of high concentration of serum albumin.

Crosslinking. When macromolecules become crosslinked they are eventually converted into a gel network in which a number of molecules are joined together so that they can no longer dissolve in solvents in which they were originally soluble. A certain number of crosslinks must be produced before gel is formed and consequently a minimum amount of reaction is necessary before any gel can be isolated. At the point where some gel begins to be detectable (the "gel point") an average of one

crosslink will have been formed per macromolecule present.[11,12] Since the distribution crosslinks is statistical this means that a small proportion of molecules will have undergone several reactions—and this gives rise to gel—while others will not have reacted at all. Once the gel point is passed the amount of gel formed is an exponential function of the amount of crosslinking.

After treatment with the bifunctional alkylating agents the sperm heads still appear to disperse normally in 2 M sodium chloride, but subsequent centrifuging shows that some of the DNA from the nucleoprotein only swells in the salt and does not truly disperse (i.e. it is spun down on high speed centrifugation).

Figure 2 shows the amount of this DNA gel formed with different amounts of CB. 3025 since this mustard is very effective in producing crosslinks the existence of a

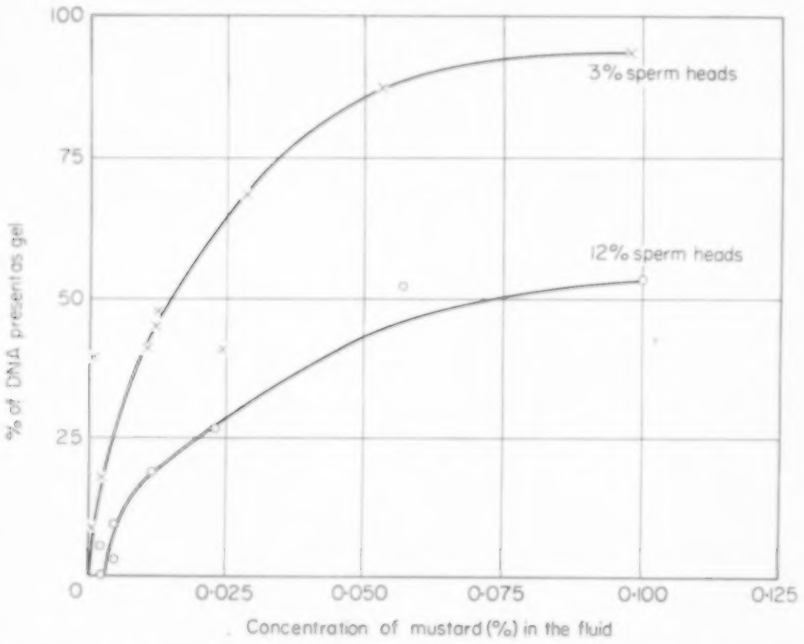


FIG. 2. Treatment of sperm head suspensions (at two concentrations) with the aromatic nitrogen mustard CB. 3025. Time of reaction at 37°C, 5½ hr during which 87·5 per cent of the mustard will have combined or hydrolysed.

threshold dose below which no gel is formed is not very clear, but with the mustard CB. 1348 (see Fig. 3) which has a much lower competition factor (i.e. a much greater proportion is hydrolysed) a gel point is clearly apparent.

Proof that the DNA, which only swells but does not truly dissolve in 2 M salt, is in a crosslinked gel network and the insolubility is not due to some other chemical reaction is provided by reactions with monofunctional nitrogen mustards which do not produce any significant quantity of gel. Apart from the inability to form crosslinks the monofunctional compounds react in exactly the same way as their bifunctional analogues and will alkylate the same groups in the nucleoprotein. If the appearance of gel were not due to crosslinking, but the result of modifying some of the polar

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groups of the molecules this change should be brought about equally by the monofunctional compounds. The monofunctional compounds examined are listed in the lower half of Table 4.

TABLE 4. CROSSLINKING OF 3 PER CENT SUSPENSION OF SPERM HEADS BY DIFFERENT METHODS

Mustard	Conc. (%)	DNA present as gel (%)
CB. 1385	0·026	84
	0·008	61
HN2	0·0098	27
HN2	0·05	74
CB. 1045	0·094	94
CB. 3039	0·033	63
CB. 1644	0·032	48
Monofunctional		
HN1	0·11	No detectable gel
CB. 1610	0·054	No detectable gel
CB. 3135	0·121	No detectable gel

The consistency of gel produced varies both with the extent of reaction and the type of crosslinking agent used. Initially, the gel is very lightly crosslinked and therefore swells greatly and its removal requires long centrifugation. With greater amounts of reaction the gel can be spun down more easily, but is still so highly swollen that it cannot be readily distinguished by eye from the viscous solutions given by the untreated nucleoprotein.

The gel produced by CB. 1348 is always much more highly swollen than that formed by CB. 3025 presumably because the latter introduces a positively charged residue which tends to collapse the negatively charged chains of DNA, whereas the reverse is the case for the negatively charged CB. 1348.

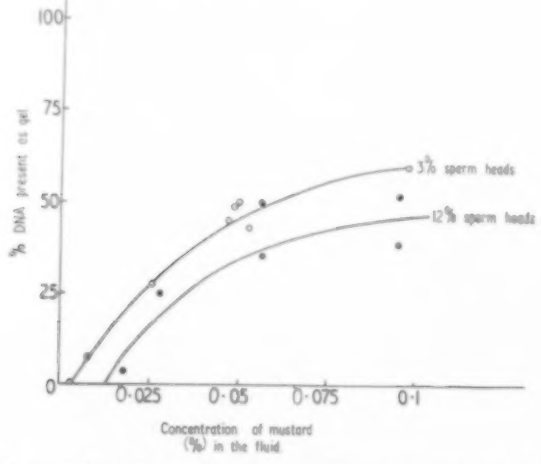
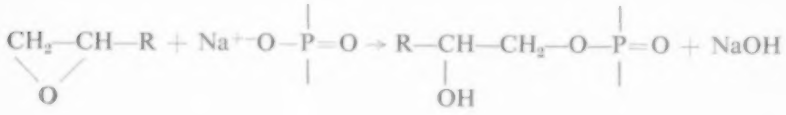


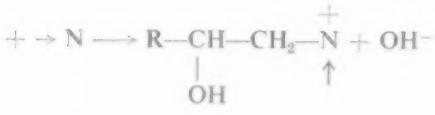
FIG. 3. Treatment of sperm head suspensions (at two concentrations) with the aromatic nitrogen mustard CB. 1348. Time of reaction at 37°C, 2 hr. during which 94 per cent of the mustard reacted (either by combination with sperm heads or by hydrolysis).

Comparison of different nitrogen mustards. All the bifunctional compounds formed crosslinks and gave rise to gel, but their efficiency varied. No compounds were examined in the same detail as CB. 3025 and CB. 1348 (see Figs. 2 and 3) although some idea of the relative efficiencies can be gained from the figures shown in Table 4.

Reaction with ethylene imine, epoxide and methane sulphonoxy derivatives

The general reactivity of these compounds is very similar and their closely related biological activity can probably be ascribed to the fact that they alkylate the same centres in the cell[4] yet the kinetics of their reactions are entirely different;[13] in aqueous solution the mustards react at a constant rate and the presence of a solute capable of alkylation merely influences the proportion which is hydrolysed. Since all the mustard used in the previous section had half reaction times which ranged from 28 to 107 min at 37°C they were almost entirely (i.e. greater than 87 per cent) consumed during the reaction with the nucleoprotein. The ethyleneimines and epoxides on the other hand are hydrolysed only very slowly by water and their rate of alkylation is proportional to the concentration of the substances with which they react. The proportion which reacts in a given time will therefore vary from experiment to experiment. In principle the amount of reaction of the ethylene imines and epoxides with the nucleoprotein might be followed by the amount of alkalinity produced since





In practice, however, the amount of reaction with the nucleoprotein is too small to be detected in this way. Because of the high molecular weight of DNA the number of crosslinks needed to convert 50 per cent into gel would result in the release of only 0·2 ml of 0·01 N NaOH in a typical experiment using 20 ml of a 3 per cent suspension of herring sperm.

Ethyleneimines. One member (CB. 1263) only of this class of compounds was examined, but it proved to be an exceptionally effective crosslinking agent. In the experiments shown in Fig. 4 no formation of alkali could be detected and this means that, at the most, only 0·1 mg could have reacted. On this basis this bifunctional ethyleneimine is a better crosslinking agent than the most effective nitrogen mustard. Again none of the protamine was involved in crosslinking and even when 90 per cent of the DNA was sedimented as a gel pellet, all of the protamine was in the supernatant.

Epoxides. Relatively high concentrations of epoxides have to be used before gel formation can be observed after a reaction period of a few hours. Ross[14] has shown that the reactivity of the epoxides is, in general, much less than that of the mustards and ethylene imines, but, the very high concentrations that are necessary before reaction with the nucleoprotein can be detected suggests that the rate of reaction with the latter

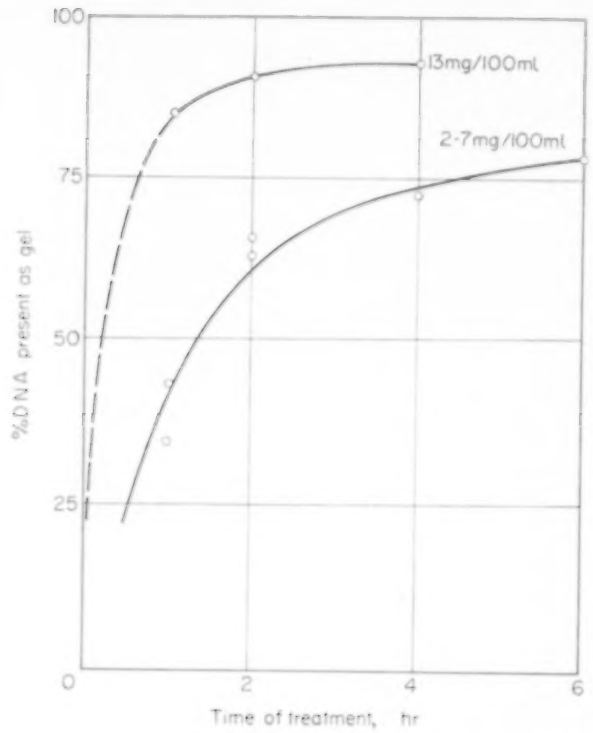


FIG. 4. Treatment of a 3 per cent suspension of sperm heads with the bifunctional ethylene imine CB. 1263. (Extent of reaction of alkylating agent could not be estimated).

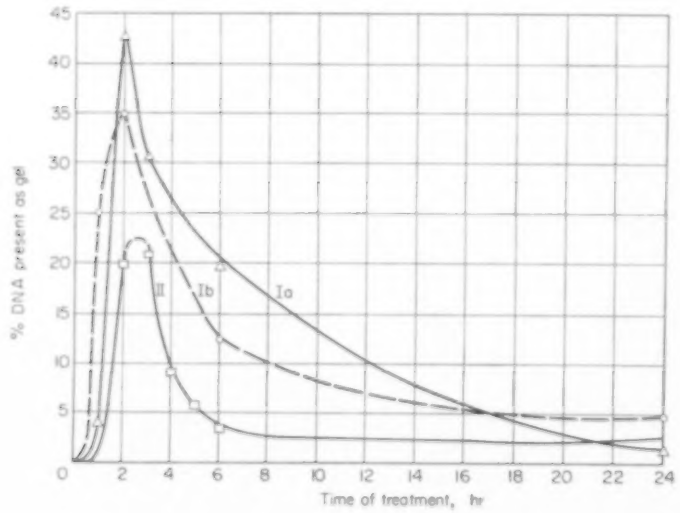


FIG. 5. Treatment of a 12 per cent suspension of sperm heads with a solution containing 4·5 per cent of bisepoxides. Curves I(*a*) and I(*b*) duplicate experiments using CB. 1161. Curve II using CB. 1170.

is surprisingly low. Another unusual feature is that the crosslinks formed appear to be unstable and with further reaction the gel fraction decreases (see Fig. 5). This behaviour has not been observed with any of the other classes of the alkylating agents examined and no explanation can be offered for the abnormal behaviour of the epoxides.

An after effect in crosslinking can be detected (see Table 5) by washing the sperm heads free of epoxides and then suspending them 5 hr later in 2 M sodium chloride in

TABLE 5. DELAYED CROSSLINKING OF SPERM HEADS* TREATED WITH BISEPOXIDES

Treatment	DNA present as gel† (%)		
	Immediate	Standing at room temp. for 4 hr	for 24 hr
4% *CB.* 1161 for 10 min	0	28·6	—
4% *CB.* 1161 for 30 min	1·5	50·8	5·0
4% *CB.* 1161 for 2 hr	42	77	6·8
4% *CB.* 1170 for 1 hr	5·4	26	—

* A suspension of 12 per cent nucleoprotein was used.
† After treatment the sperm heads were spun down and washed three times with 0·001 N NaCl. They were then dissolved in 2M NaCl either immediately or after standing.

TABLE 6. CROSSLINKING OF 3 PER CENT SPERM HEAD SUSPENSIONS BY ESTERS OF METHANE SULPHONIC ACID

Treating soln.	Amount of gel after different times of reaction (%)		
	1 hr	6 hr	24 hr
1·6% myleran	2	7	21
2·5% myleran	8	19	44
* 0·25% dimethyl myleran	29	—	—
Monofunctional			
2% *CB.* 1528	—	—	3

* Reacted for 2 hr when more than 90 per cent had reacted either with the water or the nucleoprotein.

the normal manner. Possibly one arm of the bifunctional compound reacts first leaving a second epoxide group attached to the nucleoprotein and the crosslink is only established when this has reacted.* With the more rapidly reacting reagents the crosslinking process cannot be resolved into two successive reactions.

Myleran. This substance resembles the mustards in releasing acid on hydrolysis, but its reaction kinetics are more akin to the epoxides and ethyleneimines since the rate depends on the concentration of the substrate. As myleran is not very soluble in water the reaction was carried out in 50 per cent (v/v) acetone and as can be seen from Table 6 crosslinking was obtained. No gel was produced by the corresponding monofunctional compound.

*Once the nucleoprotein has been suspended in concentrated sodium chloride this second group will react with a chloride ion in preference to the nucleoprotein. When the sperm heads are allowed to stand for some time before dispersal, the second arm can then react with another group in the nucleoprotein.

The kinetics of reaction of dimethyl myleran are like those of the nitrogen mustards and its rate of reaction is independent of the concentration of substrate. At 37°C half of it reacts in 28 min. Although more than 90 per cent is lost by hydrolysis when a 3 per cent suspension of sperm heads is treated some of it reacts to form crosslinks.

DISCUSSION OF RESULTS

Although there were good biological reasons for believing that the alkylating agents react with DNA, this was not supported by *in vitro* experiments when only some of the active compounds were found to react with DNA. Thus, while the nitrogen mustard CB. 3025 reacted readily, another highly active compound of this type, CB. 1348, did not, probably because it carried a negative charge which repelled it from the negatively charged DNA.[5, 10] In the more realistic model experiments described in this paper the DNA in the nucleoprotein was crosslinked by all the bifunctional radiomimetic alkylating agents examined. There is no point in attempting to establish a close correlation between crosslinking efficiency of any one nucleoprotein and biological activity since there are quantitative differences between different nucleoproteins. For example, the DNA nucleoprotein from mouse spleen cells is more readily crosslinked by CB. 1348 than is sperm head nucleoprotein.[18] In general, however, the order of effectiveness for crosslinking follows the same pattern as the biological activity.

The concentration of the agents necessary to produce measurable crosslinking is very low for the most effective compounds and on quantitative grounds this reaction would be expected to occur to a significant extent at the dose level at which these compounds are effective as growth inhibitors in the intact animal. It must be stressed that when gel becomes first detectable a very far reaching amount of reaction has already occurred since, *on the average*, one crosslink per molecule[11] has to be formed before any gel is formed. For example, 10 per cent of all the DNA molecule will have taken part in crosslinking when one-sixth of the amount of mustard necessary to reach the gel point has reacted. In addition to the formation of crosslinks between different molecules intramolecular reaction in which two groups of the same molecule are linked will also occur. A rough estimate of the relative frequency of inter- to intramolecular reaction can be obtained using the relationship[11, 12] that when gel formation sets in an average of one crosslink is formed for each macromolecule. With CB. 3025 gel point is reached when ten molecules have reacted with every DNA molecule (assumed to have a molecular weight of 6×10^6). The suggestion that there are many more intra- than inter-molecular crosslinks is not unreasonable and this has been found when synthetic polymers are treated with crosslinking agents.[15]

General considerations suggest that joining DNA molecules together is a most powerful method of interfering with their biological function. Other types of reaction such as substitution or the breaking of the main polynucleotide chain may effect a non-essential part of the macromolecule and thus a proportion of such reactions would be harmless. But it is most likely that the crosslinked molecules will no longer be able to fulfil their normal biological function because of the profound change in physical properties which are introduced by an inter-molecular crosslink. For loss of activity an extent of crosslinking far less than that needed to form a gel is probably sufficient, and this would suggest that something like one-fifth or one-tenth of the amount of mustard necessary to produce some gel will be sufficient to inactivate the nucleoprotein.

With the mustard CB. 3025 the threshold gel dose is approximately 0·15 mg/g of nucleoprotein and this reaction with the nucleoprotein *in vitro* was not affected by the presence of extra protein in solution (see Table 3). The same mustard inhibits the growth of the Walker tumour in the rat at a dose of 1 mg/kg of animal,[16] a dose at which a significant amount of crosslinking would be expected to be produced.

The experiments reported in this paper provide direct support for the theory[2, 3] that the polyfunctional alkylating agents exert their powerful cytotoxic action by the crosslinking of vital cell structures and indicate that the important point of attack is the DNA of the cells in the resting stage. Subsequent mitosis would either be impossible or rendered abnormal by preventing the equal sharing of the DNA complement if this has been crosslinked. This hypothesis cannot usefully be extended to all the different biological effects produced by the alkylating agents. For example, it would be difficult to see how a crude reaction of this kind would bring point mutations. It is relevant in this connexion that polyfunctionality does not always increase the mutagenicity of the alkylating agents (see review by Alexander and Stacey[17]) and that many monofunctional compounds have been found to be powerful carcinogenic agents.[19]

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PURIFICATION OF *TRANS*-N-GLYCOSIDASE OF *THERMOBACTER ACIDOPHILUS:* INHIBITION OF ENZYME BY 6-AZATHYMIDINE*

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Abstract—*Trans*-N-glycosidase from *T. acidophilus* was purified 52-fold. The enzyme catalyses the transfer of deoxyribose from thymidine to adenine, guanine, hypoxanthine, xanthine, 6-mercaptopurine, 8-aza-adenine, and 8-azaguanine. Uric acid, 2:6-diaminopurine, and 8-azaxanthine were inactive as acceptors. Thymidylic acid did not serve as a deoxyribose donor in the exchange reaction with adenine. 6-Azathymidine competitively inhibited the transfer of deoxyribose from thymidine to adenine, but had no effect on the reverse reaction and did not itself function as a donor of deoxyribose; 6-azathymine had no effect on either reaction.

THE presence of *trans*-N-glycosidase, an enzyme capable of transferring deoxyribose from one purine or pyrimidine to another, was first demonstrated by MacNutt[1] in several species of lactobacilli. One of these organisms, *Thermobacter acidophilus* (R26), has been used extensively as an assay organism for deoxynucleosides and deoxynucleotides. Prusoff and Welch[2] in a study of the antimetabolite, 6-azathymine, showed that the deoxyriboside, 6-azathymidine, is 1300 to 2000 times as effective as 6-azathymine in inhibiting the growth of *T. acidophilus* grown on a deoxyriboside-supplemented medium. This paper describes the isolation and partial purification of *trans*-N-glycosidase from *T. acidophilus*, the competitive inhibition of the enzyme by azathymidine, and various other properties of the enzyme.

METHODS

Materials

The following materials were obtained from commercial sources: thymine, uracil, cytosine, 6-mercaptopurine, 8-aza-adenine, 8-azaguanine, xanthine, 2 : 6-diaminopurine, 8-azaxanthine, guanine, and colchicine (Nutritional Biochemicals Corp.), thymidine, calcium thymidylate, cytidine and deoxycytidine (California Corporation for Biochemical Research), deoxyadenosine and *p*-chloromercuribenzoate (Sigma Chemical Co), adenine and hypoxanthine (Francis Earle Laboratories), deoxyuridine (Schwarz Laboratories), and uric acid (Fisher Scientific Co.). Thymidylic acid was prepared from the calcium salt by passage through a Dowex-50 column (H^+). 6-Azathymine and 6-azathymidine were a gift of the Lederle Laboratories Division of the American Cyanamid Co. Thymine-^{14}C was a gift of Dr. William Prusoff.

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Analytical procedures

Protein was determined by the method of Lowry, *et al.*[3] For routine assay of enzymic activity the exchange of deoxyribose between thymidine and adenine was selected. The formation of thymine was followed with a Beckman Spectrophotometer, Model DU, by measuring the increase in ultra-violet absorption at 300 mμ at pH 13.* After incubation of 2 ml of the reaction mixture for the desired time, 2 ml of a pH 13 buffer (0·5 M K_2HPO_4–0·54 M KOH) were added. Under these conditions an optical density increase of 0·380 represented the formation of 0·4 μmoles of thymine (0·1 μmole/ml). In a similar manner, reactions between purines and other pyrimidine deoxyribosides could be followed, since the deoxyribosides absorb less ultra-violet light at 300 mμ in alkali than do the free pyrimidines. These determinations were based on the fact that at this wavelength and pH, the u.v.-absorption of adenine and deoxyadenosine are the same. It was assumed that there was no difference between other purines and their deoxyribosides. The identity of the products of the thymidine–adenine reaction was confirmed chromatographically in the Na_2HPO_4–*iso*amyl system[4] and the reliability of the assay confirmed by use of thymine-^{14}C in the reverse reaction between thymine and deoxyadenosine. The specific activity of the thymidine formed, when the substance was separated chromatographically and eluted, was in good agreement with the loss of thymine determined spectrophotometrically. One unit of enzyme was defined as the amount necessary to give an optical density change of 0·380 at 300 mμ at pH 13 under the following conditions: 2·0 μmoles of thymidine, 0·4 μmoles of adenine, 0·6 ml of citrate buffer (0·05 M; pH 5·0), 0·1 ml of enzyme and water to make 2·0 ml; incubation period: 1 hr at 37°C.

PURIFICATION OF ENZYME

T. acidophilus (ATCC 11506) was maintained on Bacto-Micro Inoculum Broth (Difco). Micro Inoculum Broth (30 l.) was inoculated with 50 ml of a 30 hr culture of the organism. After 33 hr of incubation at 37°C, the cells were harvested in a Sharples Super Centrifuge; the yield, per litre of medium, was 1·5 g (wet weight) of cell paste. Portions (3 g) were washed twice with 20 ml of water, centrifuged at 2250 × *g* for 20 min at 4°C, and stored at − 15°C. Aliquots of each subsequent purification step were retained.

Cell-free extracts were prepared by disruption in a Nossal mechanical shaker.[5] Batches of cell paste (1 g) were mixed with 10 g of fine glass beads† and 9 ml of water and shaken in the cold for four periods of 20 sec each with intermittent cooling in ice. The supernatant material decanted from the cylinders and two 2 ml washings of the beads were pooled and centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant fraction was decanted ("crude extract") and the residue discarded.

The crude extract (255 ml) was treated with a solution of $MnCl_2$ (1 M; 0·05 vol.) in the cold, stirred for 30 min, and centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant fluid was repeatedly dialysed in the cold against 15 l. of distilled water (changed daily for 5 days). The extensive dialysis was necessary to remove Mn^{2+} which interfered in the protein determination and produced a brown precipitate in the assay procedure. The dialysate was centrifuged at 20,000 × *g* for 20 min at 4°C and the precipitate discarded.

* This assay method was suggested by Dr. W. H. Prusoff.

† Superbrite, Type 120. Minneapolis Mining and Manufacturing Co., St. Paul, Minnesota.

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The supernatant fluid (260 ml) was treated in the cold with a saturated solution of ammonium sulphate (508 ml) to give two-thirds saturation, and centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant material was discarded and the precipitate (ammonium sulphate I) was dissolved in cold water (50 ml) and dialysed overnight in the cold in the rocking dialyser. The dialysate was centrifuged at 20,000 × *g* for 20 min at 4°C, and a small precipitate was discarded.

The supernatant fluid (65 ml) was treated with citrate buffer (0·05 M; pH 5·0; 56 ml) and water (48 ml) in the cold; the final concentration of protein was 2 mg/ml. Ethanol (169 ml) at −20°C was added slowly with stirring, the temperature not being allowed to rise above 0°C, and the mixture was centrifuged at 20,000 × *g* for 20 min at 1°C. The precipitate was discarded, and the supernatant fraction was divided into ten portions, each of which was dialysed in the cold against distilled water (6 l. changed every hour for 5 hr, then against 15 l. in a rocking dialyser overnight). The dialysate was centrifuged at 20,000 × *g* for 20 min at 4°C and the precipitate discarded.

The supernatant material (640 ml) was treated in the cold with solid ammonium sulphate, 404 g, to give approximately 90 per cent saturation, stirred for 1 hr, and centrifuged at 20,000 × *g* for 20 min at 4°C. The precipitate was collected, dissolved in water (18 ml), dialysed against water (15 l. in the rocking dialyser overnight at 5°C), and centrifuged at 20,000 × *g* for 20 min at 4°C. The precipitate was discarded, and the supernatant solution (ammonium sulphate II) was stored as purified enzyme. A 52-fold purification was achieved (Table 1). The loss in activity observed after several months of storage in the freezer was negligible, but repeated freezing and thawing gradually diminished the activity of the enzyme.

TABLE 1. PURIFICATION OF *trans*-N-GLYCOSIDASE

Step	Total units*	Specific activity (units/mg)
Crude extract	835	0·606
Mn^{2+} fraction	749	1·29
Ammonium sulphate I	736	2·53
Ethanol fraction	303	18·1
Ammonium sulphate II	163	32·0

* Under the standard assay conditions. Computed exclusive of aliquots retained from each step.

RESULTS

Specificity of enzyme

The purified enzyme, in contrast to the crude extract, was free of nucleoside hydrolase and phosphorylase activity (assayed by incubation with thymidine alone). The enzyme is apparently specific for transfer of deoxyribose; thus, the substitution of cytidine for deoxycytidine produced no change in optical density. In addition to adenine, several other purines were found to accept deoxyribose from thymidine, e.g. xanthine, guanine and hypoxanthine; uric acid and 2 : 6-diaminopurine were inactive (Table 2). These results correspond to the purine specificity found by MacNutt[1] with the enzyme from *Lactobacillus helveticus*. In addition, the antimetabolites 8-aza-adenine and 6-mercaptopurine were active as acceptors of deoxyribose; 8-azaguanine reacted very slowly, while 8-azaxanthine failed to serve as an acceptor (Table 2).

TABLE 2. ABILITY OF DIFFERENT PURINES TO ACCEPT DEOXYRIBOSE FROM THYMIDINE

Purine	Relative activity compared with adenine	Time of incubation (min)
Adenine	1·00	60
Xanthine	1·48	60
Hypoxanthine	0·78	60
Guanine	0·56	30
Uric acid	0·05	30
2:6-Diaminopurine	0·00	30
6-Mercaptopurine	0·56	60
8-Azaguanine	0·09	60
8-Azaguanine	0·11	120
8-Azaguanine	0·12	180
8-Azaxanthine	0·08	60
8-Azaxanthine	0·00	120
8-Azaxanthine	0·00	180
8-Aza-adenine	1·06	60

The reaction mixture contained 0·4 μmoles of purine base (except uric acid, of which 0·2 μmoles was used), 2 μmoles of thymidine, 0·6 ml of citrate buffer (0·05 M; pH 5), 0·1 ml of enzyme, and water to make 2 ml.

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Formation of 6-mercaptopurine deoxyriboside

6-Mercaptopurine (6 μmoles in 1·2 ml), thymidine (6 μmoles in 0·6 ml), citrate buffer (0·05 M; pH 5·0; 0·6 ml), and the enzyme solution (0·6 ml) were incubated for 24 hr at 37°C. Of this reaction mixture, 0·25 ml was chromatographed on each of three sheets of Whatman no. 1 paper in the butanol–water–ammonia descending system[3] with appropriate controls for 30 hr (allowing the solvent to run off the paper). Under these conditions the thymine and thymidine were separated from the other components of the reaction mixture. One chromatogram, treated with cysteine–H_2SO_4[6] and heated to 85°C for several minutes, showed a pink spot indicative of deoxyribose at R_F 0·10 in addition to that of thymidine at R_F 0·50; an R_F of 0·10 corresponds to 6-mercaptopurine in this system. Separation of 6-mercaptopurine and the material containing deoxyribose was effected by chromatography in the ascending Na_2HPO_4–*iso*amyl alcohol system.[4] There was an area of yellow-green fluorescence under ultra-violet light at an R_F of 0·64; on treatment with cysteine–H_2SO_4, this area became pink. 6-Mercaptopurine, with its characteristic yellow-green fluorescence, had an R_F of 0·46. The deoxyriboside exhibited an absorption peak at 322 mμ when eluted with 0·1 N HCl and read against a paper blank eluted in the same manner. When the deoxyriboside was heated in 0·1 HCl for 5 min at 100°C and rechromatographed in the Na_2HPO_4–*iso*amyl system, two spots appeared on exposure to ultra-violet light; one of these corresponded to 6-mercaptopurine and the other to hypoxanthine. However, 6-mercaptopurine eluted from paper and treated identically also produced these two spots. It is concluded tentatively that 6-mercapto-purine deoxyriboside is formed in the reaction and that a second compound, probably hypoxanthine, was produced as an artifact in the subsequent treatment carried out to established its identity.

Thymidylic acid as a substrate

No change in optical density was observed when thymidylic acid was incubated alone or with adenine, using the purified enzyme with and without phosphate and Mg^{2+}. However, on incubation of the crude extract with thymidylic acid alone or with

adenine, a small increase in optical density was noted, indicative of a probable breakdown to thymine; this was enhanced by Mg^{2+} and phosphate ions.

Effect of metal ions, phosphate buffer, colchicine, and p-*chloromercuribenzoate*

The rate of reaction between adenine and thymidine was not affected by replacement of the citrate buffer with phosphate buffer (0·05 M; pH 5·0) or by the addition of *p*-chloromercuribenzoate (10^{-5} to 10^{-3} M), magnesium chloride (10^{-4} or 10^{-3} M), manganese chloride (10^{-3} M), (ethylenedinitrilo)-tetra-acetate (10^{-2} M), or colchicine (5×10^{-5} or 5×10^{-4} M).

Relationship of enzyme activity to pH

Early work using crude extracts indicated a pH optimum of 5·0, and all subsequent assays were performed at this pH. A repeat determination near the conclusion of this work using purified enzyme indicated that maximal activity was obtained at a pH of about 6·5 (Fig. 1).

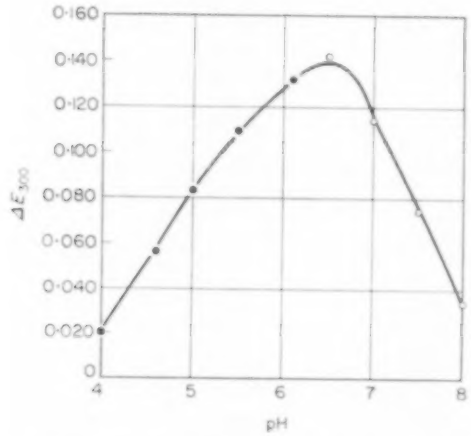

Fig. 1. Effect of pH on enzyme rate: ●, citrate buffer (0·05 M); ○, phosphate buffer (0·05 M). The reaction mixture contained 2 μmoles of thymidine, 0·5 μmoles of adenine, 0·6 ml of buffer, 0·1 ml of enzyme, and water to make 2 ml; incubation was for 1 hr at 37°C.

Search for enzyme in other sources

Cell-free extracts of *Lactobacillus casei*, obtained from Dr. C. Carter, demonstrated no *trans*-N-glycosidase activity when assayed as described. Also, a chicken kidney homogenate, obtained from Dr. E. Landon, and an homogenate of regenerating rat liver, obtained from Dr. R. Montsavinos, were without activity.

Inhibition of enzyme by azathymidine

The effect of varying concentrations of thymidine, with and without azathymidine, on the rate of reaction with adenine was studied. When the data are plotted according

to the method of Lineweaver and Burk,[7] it is seen that azathymidine acts as a competitive inhibitor of thymidine (Fig. 2). Under these conditions the K_M for thymidine was computed to be $2{\cdot}9 \times 10^{-4}$ M. Preincubation of the enzyme with azathymidine produced the same degree of inhibition. Azathymidine had no effect on the rate of transfer of deoxyribose from deoxyadenosine to thymine under analogous

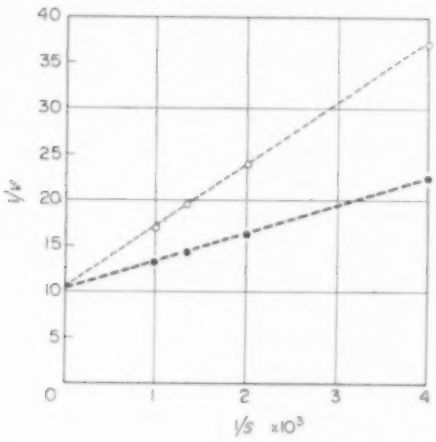

FIG. 2. Effect of substrate concentration on enzyme rate: ●, varying concentrations of thymidine (0·25, 0·50, 0·75, 1·0 μmoles/ml); ○, addition of 0·5 μmoles of azathymidine/ml. The reaction mixture contained 0·5 μmoles of adenine, varying amounts of thymidine, 0·6 ml of citrate buffer (0·05 M, pH 5·0), 0·1 ml of enzyme, and water to make 2·0 ml; incubation was for 1 hr at 37 °C. The experiment was repeated with 1·0 μmole of azathymidine added.

conditions. Incubation of the enzyme with adenine (0·25 μmoles/ml) and azathymidine (in concentrations of 1, 2 and 4 μmoles/ml) produced no change in optical density, showing that no azathymine was formed by transfer of deoxyribose to adenine. Azathymidine also inhibited the transfer of deoxyribose from deoxycytidine or deoxyuridine to adenine (Table 3).

Effect of azathymine

Azathymine was without effect on the transfer of deoxyribose between thymidine and adenine at concentrations of two and three times that of thymidine and on the reverse reaction at concentrations two and four times that of thymine.

DISCUSSION

The enzyme *trans*-N-glycosidase may be of importance in DNA-synthesis in some organisms. The lack of nucleoside hydrolase activity in the purified enzyme fraction from *T. acidophilus* makes it clear the hydrolase and transfer activity do not reside in the same enzyme. This is in contrast to evidence from studies of similar enzymes in other organisms.[8, 9]

Table 3. Effect of varying concentrations of azathymidine on transfer of deoxyribose to adenine from thymidine, deoxycytidine, and deoxyuridine

Ratio	Inhibition (%)
Azathymidine : thymidine	
1 : 2	20
1 : 1	24
2 : 1	43·5
3 : 1	47
Azathymidine : deoxycytidine	
1 : 1	10
2 : 1	15
Azathymidine : deoxyuridine	
1 : 1	20
2 : 1	31·3

The reaction mixture contained 0·4 μmoles of adenine, 2·0 μmoles of pyrimidine deoxyriboside, 0·6 ml of citrate buffer (0·05 M; pH 5·0), 0·1 ml of enzyme, varying amounts of azathymidine, and water to make 2·0 ml. The tubes containing thymidine were incubated for 1 hour; those containing deoxycytidine and deoxyuridine for 2 hr at 37° C.

Azathymidine competitively inhibits the transfer of deoxyribose from pyrimidine deoxyribosides to adenine *in vitro*, but does not inhibit *trans*glycosidation from deoxyadenosine to thymine. It is of interest to note that no activity was observed when azathymidine was used as a substrate in the exchange reaction with adenine. The inhibition of the growth of *T. acidophilus* by azathymidine was shown by Prusoff and Welch[2] to be of the competitive type and 1300 to 2000 times more effective than the inhibition produced by azathymine. These studies also showed that the organism grown on thymidine is less sensitive to azathymidine than when grown on deoxycytidine or deoxyuridine. This is in contrast to the enzyme inhibition found *in vitro* in this study (Table 3), but this may be due to the different concentrations of substrate used, the effect of other enzymes, or factors of cell permeability, such as, for example, greater interference with the transport of azathymidine into cells by thymidine than by the other deoxyribosides.

Azathymidine is more effective than azathymine as a growth inhibitor of *Streptococcus faecalis*. Washed *S. faecalis* cells were used by Prusoff[10] in the preparation of azathymidine from azathymine and thymidine, indicating the presence of a deoxyribose-transferring enzyme in this organism. Although he found that azathymine is incorporated into the DNA of *S. faecalis*,[11] presumably by prior production of azathymidine, evidence was presented to indicate that cellular death did not necessarily depend on this incorporation.

The growth of *Leuconostoc citrovorum* (later identified as *Pediococcus cerevisiae*), an organism in which MacNutt was unable to demonstrate *trans*-N-glycosidase activity,[1] is not inhibited by azathymine or azathymidine.[12] However, in the present study the enzyme was not detected in *L. casei*, which is quite sensitive to azathymine and azathymidine.[12, 13] Accordingly, it cannot be concluded that inhibition of this enzyme is the only mechanism involved in the inhibition of bacterial growth by azathymidine.

The apparent transfer of deoxyribose to the purine analogues 6-mercaptopurine, 8-aza-adenine, and 8-azaguanine is of considerable interest because of the widespread

use of these compounds for inhibition of cell growth. If by analogy with 6-azathymidine[2] and 6-azauridine,[14] the nucleosides of these compounds prove to be more effective than the parent bases as antimetabolites, then the mechanisms of their synthesis become important. 6-Mercaptopurine deoxyriboside was first prepared by Friedkin[15] from di*cyclo*hexylammonium deoxyribose-1-phosphate and 6-mercaptopurine, using horse liver nucleoside phosphorylase. The deoxyriboside of 8-azaguanine was tentatively identified by Smith and Matthews[16] in hydrolysates of deoxyribonucleic acid from *Bacillus cereus* grown on 8-azaguanine. A recent report[8] concerning *trans*-N-glycosidase ("*trans*-N-deoxyribosylase") from *L. helveticus* purified elevenfold describes the probable formation of the deoxyribosides of 6-mercaptopurine and 8-azaguanine; however, 8-aza-adenine was not tested and, apparently, its deoxyriboside has not yet been described.

Acknowledgements—We gratefully acknowledge the valuable advice of Dr. W. H. Prusoff and Dr. C. E. Carter of this department in many phases of this work.

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BOOK REVIEWS

Aspects actuels de la biochimie des acides aminés et des protéines. Actualités Biochimiques n°20. J. T. EDSALL. Masson, Paris, 1958. 156 pp., 45 fig., 7 tableaux.

CE VOLUME réunit en sept chapitres deux séries de conférences données par l'éminent biochimiste d'Harvard à la Sorbonne et au Collège de France sur la biochimie des acides aminés et des protéines. Il constitue un tour d'horizon extrêmement intéressant sur les connaissances actuelles dans ce domaine.

I. Les principales caractéristiques des amino acides et des peptides sont rappelées ainsi que les divers types de liaisons rencontrées dans les protéines. Les problèmes posés par l'apparition des acides aminés sur la terre sont décrits ainsi que les expériences réalisées (notamment celles de Miller et Urey) en vue d'étayer certaines théories développées à ce sujet. Les principales propriétés de l'eau, milieu naturel essentiel des protéines, sont résumées.

II. Dans ce chapitre, l'auteur étudie quelques aspects de l'équilibre acide–base pour les amino acides et les protéines en utilisant la théorie de Brönsted–Lowry. Il montre comment l'ionisation est fortement influencée par la réaction entre les groupements différents d'une molécule et illustre ce fait par l'examen détaillé de l'équilibre acide–base de la cystéine.

III. La séquence exacte des acides aminés de longues chaines peptidiques n'est encore connue que pour quelques substances: insulines diverses, β-corticotropine de porc, α-corticotropine de mouton. Les liaisons disulfures sont les principales liaisons covalentes impliquées dans les croisements entre chaines peptidiques. Les liaisons diesterphosphoriques sont parfois importantes et les liaisons croisées hydrogène entre chaines latérales participent à la stabilisation de l'ensemble de la protéine. L'auteur développe les restrictions qui ne permettent pour les protéines qu'un nombre limité de structures parmi lesquelles la forme hélicoidale (α, β et γ). Les conditions de stabilité de cette forme sont exposées. Les preuves expérimentales à ce sujet sont encore rares.

IV. Les protéines présentent une affinité élevée pour des substances très diverses. L'auteur rappelle d'abord l'équation de fixation d'une particule et les modes de représentation graphique généralement utilisés. L'étude de la fixation de plusieurs particules avec interaction entre les groupements actifs est illustrée par l'exemple du 4: methylimidazole pour les ions cuivre et zinc. Les méthodes de calcul de Scatchard et de Hearon et Gilbert sont expliquées. Une étude semblable a été réalisée pour la sérum albumine et le zinc. La liaison d'ions a un effet électrostatique important. Les anions sont particulièrement retenus par la sérum albumine et on a montré que deux catégories de groupe actifs entraient en jeu.

V. Les plasma de mammifères contiennent de 32 à 60% d'albumine. La technique d'isolement (basée sur la haute solubilité relative) est décrite ainsi que la cristallisation fractionnée de la mercaptalbumine en présence d'un sel de mercure formant le dimère. La technique de Dintzis (passage sur échangeurs d'ions) permet de déioniser complètement et d'enlever de petites quantités d'acides gras solidement fixés. La composition en acides aminés des sérum albumines humaine et bovine est presque la même. La stabilité à la chaleur et à l'urée est augmentée en présence d'anions d'acides gras à longue chaine. Les déterminations de poids moléculaire oscillent entre 65,400 et 72,000. La forme exacte de la molécule est encore incertaine, mais on admet généralement qu'il s'agit d'un ellipsoide avec un rapport axial de 3/1 à 4/1. On a aussi propsé la forme prismatique. La sérum albumine présente une structure lâche. Les divers groupes ioniques ainsi que ceux des chaines latérales subissent des interactions réciproques et peuvent réagir avec un grand nombre de substances du milieu environnant.

VI. La mercaptalbumine réagit aisément avec les mercuriels par ses groupes amine, imidazol, carboxyle et sulfhydryle suivant une séquence de trois réactions dont les vitesses sont très différentes. La diffusion lumineuse permet de déterminer la quantité de dimère en solution, la vitesse de réaction et le constante d'équilibre. L'auteur étudie ensuite la réaction avec un mercuriel organique bifonctionnel qui forme un dimère avec une vitesse élevée suivant une séquence de réactions semblable. L'élimination des acides gras de la mercaptalbumine en modifie les propriétés;

la vitesse de dimérisation par exemple diminue. La mercaptalbumine bovine semble plus pure et plus homogène que la mercaptalbumine humaine.

VII. Ce dernier chapitre, après quelques considérations sur la structure et la dénaturation de la sérum albumine, établit une comparaison d'ensemble avec l'ovalbumine. Celle-ci, soumise à des concentrations suffisamment élevées en urée subit, après un certain temps, une modification brusque, irréversible, traduisant une attaque de la molécule en plusieurs points simultanément, tandis qu'avec la sérum albumine on enregistre un gonflement immédiat et réversible.

L'ovalbumine semble maintenue d'une manière plus rigide dans sa configuration native par des liaisons encore mal connues.

La sérum albumine est plus flexible et ses dimensions augmentent aisément, dans certaines limites, à cause des ponts disulfure, rendant possible la pénétration des molécules du solvant. Les études aux RX suggèrent, pour plusieurs sections au moins, une structure du type hélicoide α, cette structure devenant en solution aqueuse plus ouverte et irrégulière. On peut supposer que la flexibilité et la possibilité d'adaptation de cette molécule est liée à ses propriétés biologiques.

L. DELCAMBE

Bases Physico-biologiques et Principes Généraux de Réanimation. H. LABORIT avec la collaboration de M. CARA, D. JOUASSET, G. DUCHESNE et G. LABORIT: Masson, Paris, 1958. 274 pp.

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THIS book is the first volume of a series to be published and devoted to stress, reanimation and hibernotherapy.

It presents the conceptions of the author and his school concerning the physiopathology and the treatment of troubles which appear during a stress.

In the first chapters, the author describes the principal physiological laws which rule the life of the cell; successively the following topics are discussed: intracellular and extracellular media and their reciprocal influence, the autoregulation of the ionic exchange system of the cell, the metabolic phenomena and the organic reaction against stress which play the principal role in the exchange of Na^+ and K^+ through the cell membrane.

On the basis of those considerations, the author discusses the different functions which are involved both in the import of elements which are necessary to the life of the cell and the export of H^+ ions out of the cell. The mechanism of the organic regulations and the reanimation which finally set up again normal cellular metabolic processes are studied. Amongst these techniques, hibernotherapy is mentioned.

This book is not really a text-book but rather the presentation of a personal theory which proceeds from the own and extensive experience of the author in this field.

E. PHILIPPOT

Pharmacologie et Pharmacothérapie Dentaire. O. NOEPPEL. Masson. Paris, 1958. 354 pp.

THIS volume of 354 pages can be divided into two equal parts. The first one is devoted to drugs which are especially used by stomatologists: local anaesthetics, topics, antiseptics, analgesics and hypnotics, general anaesthetics. The second part covers the remaining field of drugs commonly used in therapeutics, but the descriptions are less detailed. Drugs are essentially described and, in spite of the advertising published by the editor, the author cares very little about the mechanism of action of drugs. Many substances with only conjectured activity are described: this book will not help therapeutics to get out of empirism. This is an elementary guide-book for the students whose interest is centred on stomatology.

M. J. DALLEMAGNE

L'Hyperlipémie essentielle. J. GADRAT. Masson, Paris, 1958. 112 pp.

THIS book deals with a relatively rare illness which is described on the basis of some fifty authentic cases. It will mainly interest the clinician but a short chapter is devoted to the little that is known of its biochemical mechanism.

M. WELSCH

Exposés annuels de Biochemie médicale. P. BOULANGER, M.-F. JAYLE and J. ROCHE. 20e Série. Masson, Paris 1958. 248 pp., 58 figures.

THE largest part of this book is devoted to a study of the haemoglobins. The conditions leading to the first appearance on earth of porphyrins haemoglobins and related substances, is discussed by D. Rittenberg. J. Roche next describes the physicochemical properties and structure of the molecule of haemoglobin. A. Neuberger discusses the biosynthesis of aminolevulinic acid and haeme, while H. Benard, A. Gajdos and Mme. Gajdos-Török report on the biogenesis of haemoglobin in the erythrocytes of peripheral blood. Our present knowledge of the plasmatic factor which stimulates the biogenesis of haemoglobin by haemolysates of erythrocytes or reticulocytes cultivated *in vitro*, is presented by A. Nizet and C. Rimington. I. M. London, J. C. Savoie and H. Morell discuss their observations on the separate biosynthesis of haeme and globin while G. Shapira, J. C. Dreyfus and J. Kruh present a study of the lifetime of the haemoglobin molecule. The diversity of foetal, normal and abnormal haemoglobins is finally discussed, respectively, by J. Jonxis, by Y. Derrien and by V. Ingram.

This group of ten reports is therefore a complete and up-to-date survey of a subject much studied recently. The other six reports deal with various subjects. H. Lettré discusses the response to chemical agents of cells cultivated *in vitro*. K. Félix reports on the role of polypeptides in physiology and pathology. The chemistry and biological activities of lipoids from Mycobacteria and especially their possible role in tuberculosis are presented by E. Lederer. A paper by J. Brachet and another by C. de Somer give recent and pertinent information respectively on the biochemical role of the cell nucleus and on the properties of the lysosomes or cytoplasmic granules from the rat liver bearing a variety of acidic hydrolases. The closing report by J. Badin deals with the specific biochemistry of rheumatoid arthritis.

This twentieth series of the *Exposés annuels de Biochimie médicale* assumes the high standard of the preceding volumes. In addition it contains a lot of useful and up-to-date information of great interest for the scientist interested in biochemistry at the cellular and molecular level.

M. WELSCH

Pratique du Laboratoire. Edited by CH. JAULMES and J. QUERANGAL DES ESSARTS. Second Edition. Masson, Paris, 1958. 872 pp.

THIS is a compendium of the techniques in general use in the medical laboratory. They are classified under six titles: (1) general techniques; (2) biological diagnosis; (3) biochemical laboratory examinations; (4) food analysis; (5) haematology, serology; (6) parasitology, entomology.

Each method or recipe is fully described and a good analytical index enables the reader to find easily whatever he is looking for. There are, however, no full references to the original papers. Obviously the choice of some techniques and the omission of others are to some extent a matter of personal taste and cannot meet with unanimous agreement. In a few cases, introductory remarks, being oversimplified on account of lack of space, may be somewhat misleading for the unprepared mind. However, this book contains a lot of practical information in a condensed form and should be useful on the laboratory bench.

M. WELSCH

Le placenta humain. J. SNOECK *et al.* Masson, Paris, 1958. 700 pp.

THIS book is the fruitful result of the work of the medical unit of the Clinic for Gynecology and Obstetrics of the University Hospital, Brussels. It groups in an ordered synthesis the wide, but until now scattered knowledge on the morphology, biochemistry and physiology of the human placenta.

Each main chapter is divided into two parts: first, a clear, concise and complete account of acquired facts; second, a personal contribution based on experimental research.

Each in turn, Professor Snoeck's co-workers describe at length the morphology and the microscopic as well as the macroscopic aspects of the placenta, then approach its physiology from the double viewpoint of placenta permeability and of endocrine function.

A critical and analytical spirit pervades the various reports. We must also equally stress the exceptional rigour with wnich the numerous biochemical problems of this monograph are treated, as well as the density and abundance of collected data.

The authors endeavour to establish a correlation between the morphological and functional aspects of the placenta, their relationship still being imprecise They show how hazardous it is to refer to histological pictures to diagnose functional disorders. This monograph raises more problems than it provides solutions, but it is not the least of its merits.

O. GOSSELIN

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Surface Phenomena in Chemistry and Biology. Edited by J. F. DANIELLI, K. G. A. PANKHURST and A. C. RIDDIFORD. Pergamon Press, London, 1958. 330 pp., 63*s*.

THE articles in this book were written and gathered together in honour of Professor N. K. Adam who, until recently, occupied the Chair of Chemistry at Southampton University. Some eminent names figure among the contributors, who include many of Professor Adam's former pupils and colleagues. Such authors and such a title lead one to hope that here, at last, is a book which will give a clear account of the present state of affairs in surface chemistry and its main fields of application.

Yet, in this particular respect, the volume is something of a disappointment. The introductory article is a chatty, rather myopic historical survey of events in the chemistry of soaps between 1917 and 1957. When the author refers to "work carried out with too little thought, planning and care" he could almost be alluding to the book to which he is contributing. For, among some excellent review articles dealing with specific aspects of surface chemistry, there are one or two obvious pot-boilers. Then, there are included six original experimental papers which, although of interest to the specialist, seem out of place in such a compilation (one of these, on the measurement of vapour pressures, has no general relevance to surface phenomena at all). The essential weakness of the work is, therefore, an unevenness in the level of the contributions and this, perhaps, arises from uncertainty in the minds of the editors as to their potential readers.

Nevertheless, the book contains much of value for the worker in the field of pure or applied surface chemistry and for those with more general interests; students who wish to discover what types of problem confront the modern surface chemist will do well to read it.

Possibly the most satisfactory section of the work is the first, in which six authors describe the present position in the field of monolayer studies, a subject to which Professor Adam himself has contributed so much. Taken together, these articles describe the electrical and non electrical forces which operate between the molecules in surface films, the molecular associations formed as a result of such interactions and the resultant effects on the physical state and chemical reactivity of the films. Then there are two interesting articles by Stevenson and Askew which illustrate some of the problems involving surface phenomena which are encountered in industry and technology.

On the whole, the section dealing with biological phenomena is adequate, although one of the essays (on the Gibbs–Donnan equilibrium) is surely irrelevant. It would have been interesting, too, to have included something on *recent* views on membrane structure and on the mechanism of drug action. The role which the surface chemist has to play in modern biological studies has changed in the last few years. The days when he could make a really significant contribution to biology simply by studying monolayers have long passed; the modern surface chemist prefers to work with the living cell—Adams and Rideal provide a typical example of this in the present work, in their article on the

surface behaviour of *Mycobacterium phlei* as do Hartley and Brunskill in their interesting description of the manner in which water drops bounce off leaves. Yet, it must be said that the extremely readable article by Goldacre ("Surface Films, Their Collapse on Compression, the Shapes and Sizes of Cells and the Origin of Life"), speculative and qualitative though it is, is one of the most fascinating in the book.

To sum up: this book can be recommended to all those who are interested in surface phenomena. Such readers will inevitably find, however, that certain articles are too superficial, or too specialized for their taste or purpose.

F. H. TAYLOR

Amino Acids and Peptides with Antimetabolic Activity. Edited by G. E. W. WOLSTENHOLME and CECILIA M. O'CONNOR. A Ciba Foundation Symposium. Churchill, London, 1958. 286 pp., 45*s*.

THIS book consists of the seventeen papers read at a Symposium held in March 1958 under the auspices of the Ciba Foundation. The theme of the meeting was the antimetabolic and cytotoxic properties of various amino acid and peptide derivatives but the scope was widened to include several purely chemical papers as well as those with a more biological background.

There are papers concerning the elucidation of the structures of actinomycin D, etamycin, bacitracin and some cephalosporins. An account is given of the stereochemistry of naturally occurring β-amino acids. Two papers deal with synthetic methods: one concerns cyclic peptides and the other describes the amino-acyl insertion reaction. This reaction, in which peptides are built up by stepwise insertion of amino acid residues, is of considerable interest particularly as the mechanism is very reminiscent of current theories on the mode of formation of isotactic polymers. Two papers deal with oxidation products from peptides. The first discusses the formation of disulphides from cysteine peptides and the second the formation of biologically active compounds from peptides when the oxidation level of the nitrogen atom in an amide structure is raised to that of hydroxylamine.

Six papers deal with topics related to the treatment of malignant disease. There is a useful account of the influence of amino acid deficiencies on cancer chemotherapy in which some unexpected findings are mentioned, for instance 6-diazo-5-oxo-L-norleucine (DON) becomes more effective when the diet is deficient in *iso*leucine and L-penicillamine becomes an active tumour growth inhibitor if vitamin B_6 intake is restricted. One has the feeling that the relative lack of effectiveness of known amino acid antagonists—exceptions being azaserine, DON, and ethionine—may be due to inadequate knowledge regarding dietary control. Three papers deal with amino acid structures carrying a nitrogen "mustard gas" grouping; one is a general account of the chemistry and two deal with clinical results. The antitumour actions of azaserine and DON are discussed and an account is given of similar studies on β-2-thienylalanine and actinomycin D. The latter two compounds are apparently only effective at almost toxic dose levels but there is a clear challenge to organic chemists to prepare related compounds with a view to separating the toxic from the carcinolytic properties.

Finally there are contributions in which the mechanisms of action of azaserine and chloramphenicol are discussed.

The discussions which followed the various papers are reproduced in full and while the printing of such comments is not always valuable it must be admitted that in the case of this Symposium they add considerably to the value of the book as a record of a most interesting meeting.

W. C. J. ROSS

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Papers to be published in future issues

B. R. BAKER: Potential anticancer agents—II. A proposed mechanism for the anticancer action of L-azaserine and 6-diazo-5-oxo-L-norleucine.

I. BERENBLUM, D. BEN-ISHAI, N. HARAN-GHERA, A. LAPIDOT, E. SIMON and N. TRAININ: Skin initiating action and lung carcinogenesis of derivatives of urethane (ethyl carbamate) and related compounds.

P. VAN DUIJN, R. G. J. WILLIGHAGEN and A. E. F. H. MEIJER: Increase of acid phosphatase activity in mouse liver after dextran storage.

I. GRUNLAND: Structure chimique et activité oestrogène.

E. SPECTOR and F. E. SHIDEMAN: Metabolism of thiopyrimidine derivatives: thiamylal, thiopental and thiouracil.

I. BERENBLUM, B. BLUM and N. TRAININ: Failure of the urethane antagonist—lysergic acid-diethylamide (LSD-25)—to inhibit lung carcinogenesis or the initiating phase of skin carcinogenesis in mice.

B. H. MARKS, R. SORGEN and H. GINSBERG: Histamine-liberator 48/80 and basophilic leukocytes.

C. J. CAVALLITO and P. SANDY: Anticholinesterase activities of some bis-quaternary ammonium salts.

JEAN M. HAGEN: Stimulation of secretion and metabolism in mouse parotid glands *in vitro*.

F. J. LIONETTI, W. MCLELLAN and F. COMUNALE: Inhibition of oxygen consumption in human erythrocytes by hexetidine.

W. C. J. ROSS: Tumour growth—inhibitory activity of some sulphur mustard gas derivatives.

W. H. PRUSOFF: Further studies on the inhibition of nucleic acid biosynthesis by azathymidine and by deoxyadenosine.

J. COOPER and T. MARCHESI: The possible biochemical lesion in blindness due to methanol poisoning.

B. H. SELLS: Influence of 6-azauracil on the metabolism of *Bacillus cereus*.

A. MCCOUBREY: Inhibition of monoamine oxidase by 1-phenylethylamines.

V. ERSPAMER: Isolation of bufoviridine from the skin of *Bufo viridis* and its identification as dihydrobufothionine.

A. SEKERA and C. VRBA: Statistical evaluation of the relations between physicochemical properties and local anaesthetic activity.

E. D. WILLS: The effect of some organic peroxides on the sulphydryl enzymes.

T. FREDRIKSSON and G. TIBBLING: Demonstration of direct cholinergic receptor effects of methylfluorophosphorylcholines.

S. GREENBERG, T. MASURAT, E. RICE, J. HERNDON and E. VAN LOON: The effect of chlorpromazine hydrochloride on glutamic acid decarboxylase and pyridoxal kinase in rat brain homogenates.

A. W. LESSIN: The pharmacological evaluation of monoamine oxidase inhibitors.

ERRATA

V. BONAVITA and V. SCARDI, *Biochemical Pharmacology* **2**, 58 (1959)

On page 60.
In the legend to Fig. 2 "40 μmoles of Py5P (○) or Py5PINH (●)" should read "40 mμmoles of Py5P(○) or Py5PINH (●)".

WALTNER K., JR., CSERNOVSZKY M. and KELEMEN E., *Biochemical Pharmacology* **2**, 50.

On p. 53.
The caption to Fig. 1 should include:-
Effects of salicylate and DNP on salt and water diuresis of rats.

On p. 54.
The caption to Fig. 2 which was omitted should read:-
Fig. 2. Relation of doses of salicylate and of increase of oxygen consumption of normal rats
▲ adults, weighing 175–210 g
● small rats, weighing 60–120 g

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Biochemical Pharmacology, 1959, Vol. 2, pp. 161–167. Pergamon Press Ltd., London

POTENTIAL ANTI-CANCER AGENTS*—II.

A PROPOSED MECHANISM FOR THE ANTI-CANCER ACTION OF L-AZASERINE AND 6-DIAZO-5-OXO-L-NORLEUCINE

B. R. BAKER

Department of Biological Sciences, Stanford Research Institute, Menlo Park, California

Abstract—An organic mechanism of action for the enzymic activation of the amide-NH_2 group of L-glutamine as an aminating agent has been proposed, with pyridoxal phosphate acting as the coenzyme. The non-competitive inhibition of L-glutamine and L-glutamate function by L-azaserine and 6-diazo-5-oxo-L-norleucine (DON) has been attributed to irreversible alkylation of the phosphate group of the pyridoxal complex formed from these inhibitors.

L-Azaserine and DON have been classified as members of a generic class of compounds called irreversible amino acid antagonists.

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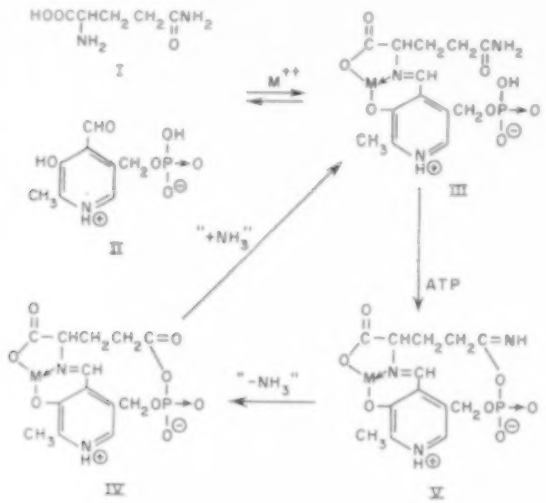
ONE of the important cofactors in enzymic synthesis at the cellular level is L-glutamine (I), which serves as an NH_2-transfer agent. In some reactions, the L-glutamine is converted to L-glutamic acid when the amide-NH_2 group is transferred;[1–7] the L-glutamic acid then reacts enzymically with an ammonia source to regenerate the L-glutamine. In other reactions, the L-glutamine transfers the α-amino group with formation of α-oxoglutaramic acid by transamination.[8–10]

The transamination reactions involving the α-amino group of L-glutamine are readily understood by the pyridoxal-invoked mechanisms described by Snell and his co-workers.[11] However, the amination reactions involving the amide-NH_2 of L-glutamine are more obscure. Since it is a well-known fact in organic chemistry that the NH_2 group of an amide does not act as an aminating agent, some activation of the carboxamide group of L-glutamine is required for this group to have aminating properties. In contrast, the imino group of an imino ether can serve as an aminating agent and, like an amine, is basic. It could be expected that an enol phosphate of the carboxamide function would give the required activation similar to an imino ether. In addition, the fact that many enzymic reactions of amino acids occur via their complexes with pyridoxal phosphate[11] can lead to consideration of this prosthetic group for meeting the activation requirements of L-glutamine.

Condensation of pyridoxal phosphate (II), L-glutamine, and a metal ion would give the reversible complex (III), that can bind to the apoenzyme through the $NH^{\oplus}$, $PO^{\ominus}$, and metal (by further chelation) functions. Ring closure of the complex (III), to an enol phosphate (V) of the amide affords a compound with aminating properties. Since such a ring closure would give a high energy compound, the reaction could be envisioned as a reaction of the complex (III) with adenosine triphosphate (ATP) to give a highly active pyrophosphate of (III) (with concurrent formation of adenosine

* This programme is under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, Contract No. SA-43-ph-1892. This paper is part of a lecture presented before the California Section of the American Chemical Society on 16 September, 1957.

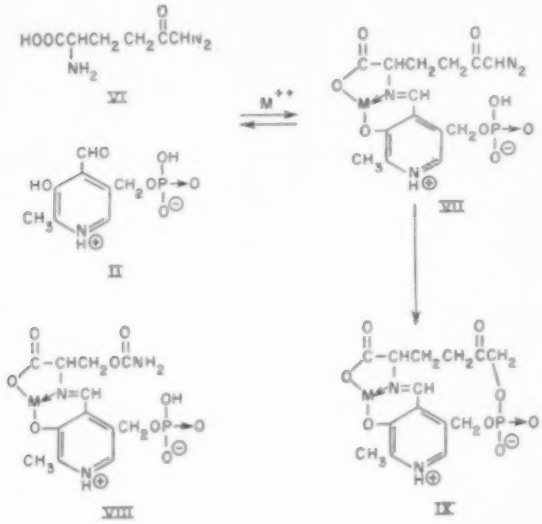
diphosphate) followed by ring closure with elimination of phosphate. The transfer of ammonia from the activated complex (V) to a substrate will form the mixed anhydride (IV). This mixed anhydride (IV) in turn will react with an ammonia source to regenerate the original glutamine complex (III) thus completing the cycle. The requirement for ATP when glutamine acts as an aminating agent[1–4] is met by the cyclization step (III)→(V). That the tetracyclic system in (IV) and (V) is feasible and strainless is readily shown with molecular models of the Fischer–Hirschfelder type.



Levenberg *et al.*[12] have shown that in the early stages of the enzymic conversion of formylglycineamide ribotide to the corresponding amidine, the antagonistic effect of L-azasermine (O-diazoacetyl-L-serine) and 6-diazo-5-oxo-L-norleucine (DON) could be competitively reversed with L-glutamine. However, after a short period of time, the enzyme system could no longer carry out this reaction, even in the presence of a high concentration of L-glutamine. Similar results were obtained if the enzyme system was allowed to react with L-azaserine or DON prior to addition of L-glutamine. These authors[12] explained the results by stating that the enzyme or an unknown coenzyme combined irreversibly with L-azaserine and DON.

With pyridoxal phosphate implicated in the aminating action of L-glutamine, it is now possible to formulate a mechanism for the action of L-azaserine[13] and 6-diazo-5-oxo-L-norleucine[14] (VI) as both competitive and noncompetitive antagonists of L-glutamine. Since DON would appear to have the same shape and electrostatic properties as L-glutamate or L-glutamine, at the α-amino acid end of the molecule, it follows that DON (or L-azaserine) could combine reversibly with pyridoxal phosphate (II) to give the chelate complex (VII). In addition, alkyl phosphates are known to react with diazoalkanes to give dialkyl phosphates.[15, 16] Thus internal alkylation of the phosphate group of (VII) with the diazomethyl group of (VII) should take place readily to give the tetracyclic ring system (IX). That such a tetracyclic system is sterically possible is again readily demonstrable with molecular models. Thus, the formation of (IX), or an analogous compound from L-azaserine, would deactivate the enzyme

system, since the antagonist (DON) could no longer be displaced from the pyridoxal phosphate moiety by L-glutamine in a competitive fashion, as in (II) ⇌ (VII), at any significant rate. Note that this is a deactivation of the coenzyme, rather than the apoenzyme.*



The observations that L-azaserine and deoxypyridoxine potentiate each other with respect to their effects on Sarcoma 180 and on Adenocarcinoma 755, as well as on *E. coli*,[17] can now be explained as a sequential block[18] of the following two reactions:

$$E + P \rightarrow EP$$
$$\quad\; P'$$

$$EP + G \rightarrow EPG$$
$$\quad\; G'$$

The amount of available enzyme–pyridoxal complex (EP) is decreased by competitive formation of enzyme–deoxypyridoxine complex (EP′). The less available EP is then competed for by the glutamine antagonist (G′), thus decreasing again the available EPG for amination reactions.

An alternate explanation to that of lack of drug absorption by the resistant cells[19] can also be made for the mechanism of resistance of mouse plasma cell neoplasm

* It should be possible to show this relationship between pyridoxal phosphate and L-azaserine (or DON) microbiologically. The growth-inhibiting properties of these antagonists should be reversible by pyridoxal phosphate, provided that cells are selected for test that can absorb pyridoxal phosphate through the cell wall. In some microbial systems the reversal of this inhibition may be possible by pyridoxine or pyridoxal provided the cells have an enzyme system that can convert these B_6- compounds to the active coenzyme, pyridoxal phosphate.

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70429 to L-azaserine. That this resistance did not involve an alternate pathway was demonstrated by the fact that cell-free extracts of this resistant line still carried out the conversion of formylglycineamide ribotide to the corresponding amidine; this isolated enzyme system from the resistant cells was just as susceptible to L-azaserine inhibition as the enzyme system from the non-resistant cells.[19] Since the same enzymic step is involved in both the resistant and non-resistant strains, it is possible that the resistant strain produces larger amounts of pyridoxal phosphate in order to reactivate the enzyme with new coenzyme. In the isolated enzyme system, new synthesis of pyridoxal phosphate is not possible and inhibition is normal. Such an explanation could be readily demonstrated by a microbiological assay for increased pyridoxal production in the resistant neoplasm compared to the sensitive neoplasm.

At first glance it is surprising that O-carbamyl-L-serine,[20] as its complex (VIII) with pyridoxal phosphate, does not have the aminating properties of L-glutamine complex (III). However, this can be rationalized on the basis that the L-glutamine complex (III) becomes active as an aminating agent only after conversion to the enol phosphate (V), a reaction which could be much more difficult for the C=O of the carbamyl group, since this carbon is at the carbonic oxidation level and formation of an enol phosphate could require much more energy than in (III)→(IV). Therefore, O-carbamyl-L-serine could be expected to be a reversible antagonist,[12, 20] albeit a poor one, by formation of a pyridoxal phosphate complex on the enzyme.

The fact that the L-isomer of azaserine is an L-glutamine antagonist but the D-isomer is inactive[12, 13, 21, 22] could be readily rationalized on the basis that only the L-isomer of the antagonist can fit the steric requirements of the enzyme system for L-glutamine. However, the fact that the D-isomer of 6-diazo-5-oxonorleucine (containing less than 0·1 per cent of L-isomer) has activity against Sarcoma 180, but to a lesser degree than the L-isomer,* shows that the above explanation for the inactivity of L-azaserine has been oversimplified. With pyridoxal phosphate implicated as a coenzyme in L-glutamine action, a more plausible explanation is now possible.

The 6-diazo-5-oxo-D-norleucine complex with pyridoxal phosphate (VII) can be converted to the L-isomer via Schiff base isomerization to the racemizing form[11] of the pyridoxal phosphate complex (X). Thus, the L- and D-isomers are interconvertible and both are active. However, when the pyridoxal complex of D-azaserine (XI) shifts to the racemizing form (XII), this form (XII) can also eliminate a β-substituent;[11] elimination can be expected if the β-substituent on the amino acid is an oxygen, sulphur, or aryl function, to give the α-aminoacrylic acid complex (XIII). If it is assumed that elimination (XII)→(XIII) occurs much more rapidly than reversion of XII back to XI, then the D-isomer of azaserine will be devoid of activity.†

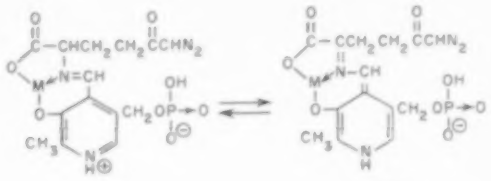
Reilly has shown that an enzyme from liver can deactivate L-azaserine,[23] but has no effect on DON.‡ Such a specificity is readily rationalized on the basis that the azaserine-deactivating enzyme also has pyridoxal phosphate as a prosthetic group. Thus, the azaserine–pyridoxal phosphate complex (XI) can decompose by β-elimination of

* Unpublished work by C. C. Stock, Sloan-Kettering Institute for Cancer Research. The writer wishes to thank Dr. Stock and Dr. A. M. Moore of Parke, Davis & Company for release of this information prior to publication.

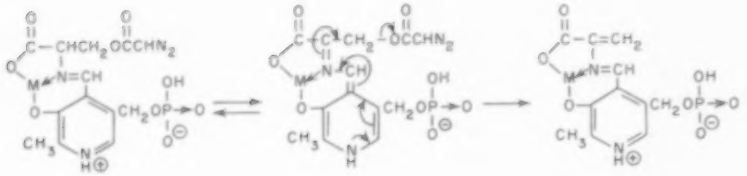
† This explanation would suggest that the 2-oxo analogue of 6-diazo-5-oxo-L-norleucine, namely, 6-diazo-2:5-dioxocaproic acid, should have a biological action similar to the amino acid antagonist by condensation of the pyridoxamine form of the coenzyme with the diketo acid to form the complex (X).

‡ Unpublished work by H. C. Reilly quoted in reference 24.

diazoacetate by the sequence (XI)→(XII)→(XIII); in contrast, the complex with DON (XII)⇌(X) cannot eliminate the β-substituent. That pyridoxal can readily decompose azaserine by non-enzymic β-elimination, but that DON is stable to β-elimination, has been recently demonstrated.[25]



VII X



XI XII XIII

Since L-glutamine and L-glutamic acid are known to be aminating agents in many enzyme systems, it could be expected that with any given L-glutamine (or L-glutamic acid) antagonist, a definite decreasing order of susceptibility for the enzyme systems should be expected. Secondly, the most susceptible enzyme system may vary with the antagonist. Such differences have been observed with L-azaserine and DON in *E. coli*.

The aromatic amino acids such as phenylalanine, tyrosine and tryptophane stand out in their ability to reverse *E. coli* inhibition.[21, 26, 27] In contrast, DON inhibition of *E. coli* was unaffected by phenylalanine, tyrosine or tryptophane, this inhibition being best reversed by purines.[27] Thus, the L-glutamine enzyme system in *E. coli* most susceptible to blockade by DON is one involved in purine biosynthesis, most probably the formylglycinamide ribotide to amidine step.[3, 4]

The enzyme systems most susceptible in *E. coli* to azaserine inhibition are obviously those involved in the synthesis of aromatic amino acids,[21, 26, 27] but the data available for interpretation are less clear than in the case of DON. If one continues with the assumptions made in this paper that azaserine competes with L-glutamine for the site on pyridoxal involved in amination reactions, then it follows that azaserine blocks the synthesis of aromatic amino acids from the corresponding α-keto acids[8] by competing for the site of L-glutamine or L-glutamic acid required in these reactions. It is more difficult to understand how phenylpyruvic acid can reverse the effect of DON,[26] since the competition for an enzyme site in the phenylpyruvate → phenylalanine reaction involves the cofactor, L-glutamine, and not the metabolite, phenylpyruvic acid. More data are needed to establish the intimate mechanism of reaction, although it is possible that some other enzyme system that does not involve glutamate or glutamine may be accelerated under the stress conditions to convert phenylpyruvic acid to phenylalanine.

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Other points of interest in this area bearing on the question of relative enzyme susceptibility are (*a*) azaserine also blocks purine synthesis *de novo*,[26, 28] but to a lesser degree than aromatic amino acid synthesis; (*b*) certain strains of *E. coli* resistant to azaserine are not cross-resistant to DON and *vice versa*.[27]

The preceding concept of relative enzyme susceptibility is of utmost importance in chemotherapy in general and in cancer chemotherapy in particular. At this stage in cancer research no gross differences in reaction paths between cancer cells and normal cells have been observed, but only a difference in degree.* Therefore, other analogues of L-azaserine and DON might preferentially inhibit other glutamate or glutamine reactions and one of these—perhaps as yet unknown—glutamate or glutamine systems may be more important to a cancer cell than a normal cell. For example, the conversion of uridylic acid to cytidylic ,involving glutamine as a cofactor,[7] is decreased by DON,[31] but this enzyme[3, 4] is probably not as susceptible to DON blockade as the enzyme converting formylglycineamide ribotide to the amidine.[27]

Since a competitive L-glutamine inhibitor[12, 20] such as O-carbamyl-L-serine is not an anticancer agent,† while the non-competitive inhibitors L-azaserine[13] and DON[14] have anticancer activity, it follows that the irreversible coenzyme deactivation reaction by these diazo-type antagonists most probably accounts for the anticancer activity, rather than mere competitive glutamine inhibition.‡§

This concept of irreversible combination of an amino acid with pyridoxal phosphate should have considerable chemotherapeutic application in the amino acid area and even in other areas such as a possibility of reduced triphosphopyridine nucleotide (TPNH)–aminopterin combination in the irreversible block of folic acid reduction. Many other reactions of amino acids other than glutamic acid and glutamine have been mechanistically described by Metzler *et al.*[11] It should be possible to put alkylating groups on other selected amino acids such as phenylalanine or tryptophane that would irreversibly deactivate the pyridoxal–apoenzyme systems or other enzymes that synthesize or utilize these amino acids. Although Woolley[34] has stated that an antimetabolite should be as close as possible in structure to the corresponding metabolite, his concept should be modified to state that the antimetabolite should be as close as possible in structure in that part of the molecule where the stereospecific requirements of the enzyme surface must be met. Certainly there can be areas on the metabolite that are away from the enzyme surface where considerable change in size can be made without influencing adsorption on the enzyme surface. This phenomenon has been demonstrated by Caughey *et al.*[35] They observed that *iso*phthalate and glutarate competitively inhibited glutamic acid dehydrogenase to the same extent, but that phthalate and terephthalate were much less effective; even 5-carboxy*iso*phthalate was an effective inhibitor. With the reasonable assumption that bonding to the enzyme occurs through

* The absence of some degradative enzymes in certain cancer cells has led Potter [29] to propose the "deletion hypothesis". The utilization of this hypothesis to design enzyme blocks that cannot be detoxified by susceptible cancer cells, missing a degradative enzyme, but can be detoxified by normal cells containing the necessary enzyme, is another approach to chemotherapy that is outside the scope of this paper. The selective detoxification of thioguanine has recently been observed by Moore and LePage.[30]

† Unpublished work by Dr. C. C. Stock, Sloan-Kettering Institute.

‡ Moore and LePage[32] have stated that "the significant *in vivo* effect of azaserine on mammalian cells involves an irreversible combination of azaserine with the cell component, presumably the enzyme involved." (See also reference 33.)

§ Note added in proof. Skinner *et. al.*[36] have recently reported on the lack of anti-cancer activity of O-carbamyl-L-serine.

the two carboxylate groups, they stated "it is apparent that the portion of the molecule orientated toward the enzyme surface is structurally much the same in both these compounds (glutarate and *iso*phthalate)"; it is obvious that the remainder of the benzene ring constitutes a considerable change in gross measurements of the anti-metabolite, particularly in the case of 5-carboxy*iso*phthalate.

It is worth while to speculate that azaserine and DON should be considered the first members of a broad class of irreversible inhibitors—many of which are yet to be discovered—that are derived from a substrate bearing an alkylating group.

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SKIN INITIATING ACTION AND LUNG CARCINOGENESIS BY DERIVATIVES OF URETHANE (ETHYL CARBAMATE) AND RELATED COMPOUNDS*

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Abstract—The possibility that a metabolite might be implicated in the carcinogenic action of urethane on the lungs, and in its limited "initiating" action in the case of skin carcinogenesis, prompted the testing of a number of derivatives and analogues of urethane for both lung carcinogenesis and skin initiation in mice. The modifications in molecular structure of urethane involved (i) changes in the carbamyl portion, (ii) changes in its carboethoxy portion, (iii) phosphorylated derivatives, and (iv) addition compounds with keto and amino acids. Most of the compounds tested proved inactive, and among those that did give positive results, the effects were weak; thus indicating that none of them represented the sought-for metabolite. Some tentative implications of the results are discussed as pointers for further enquiry.

Renewed interest in the biological properties of urethane and in its metabolic fate in the animal body arose from the fact that, in addition to its carcinogenic action on the lungs[2] and the fore-stomach[3] in mice, and on the liver and lungs[4] in rats, urethane also possesses a pronounced "initiating" action on mouse skin, either when applied locally[5, 6] or administered systemically,[7] rendering the skin of such treated animals responsive to the "promoting" action of croton oil.[1] The problem of the metabolism of urethane was further stimulated by the observation of Rogers[8, 9] on the possible participation of (unidentified) metabolites in the neoplastic transformation of lung tissue by urethane.

Attempts to determine the nature of the postulated metabolite of urethane (as distinct from the end-products, ethanol, ammonia and carbon dioxide, which are manifestly not implicated) by metabolic studies involving ^{14}C-labelled urethane, have so far been unsuccessful.[10, 11] The present investigation represents another, more empirical, approach, in which a series of urethane-related compounds that could conceivably be intermediate metabolites of the parent compound, were tested for skin initiation and for lung carcinogenesis in mice. If any one of these proved to be at least as potent as urethane itself, the possibility of it representing a key metabolite responsible for the action of the parent compound could serve as a pointer for further metabolic studies.

The choice of compounds for testing was based not only on the known hydrolytic

* A preliminary report of these findings was included in a review by one of the authors (Berenblum)[1].
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breakdown of urethane in the body, but also on possible oxidative mechanisms, as side reactions, and on addition compounds between body constituents and urethane itself or some of its postulated breakdown products. The compounds tested may conveniently be considered under four headings: (i) changes in the urethane molecule involving the carbamyl group; (ii) changes involving the carboethoxy group; (iii) phosphorylated derivatives; and (iv) addition compounds with keto or amino acids.

METHODS

The animals used in these experiments were male and female mice of the Swiss strain, bred in these laboratories by brother–sister mating for from fifteen to twenty generations. They were 2–3 months old at the start of the experiment, kept in plastic cages, housed in an air-conditioned room at 21–23 °C, and fed on *Purina Laboratory Chow* and water *ad libitum*.

The urethane derivatives and related compounds were synthesized by the following methods:

The N-methylethylcarbamate, N : N-dimethylethylcarbamate, N-hydroxyethylcarbamate, allylcarbamate, carboethoxyglycine, carboethoxy-l-alanine, carboethoxy-l-aspartic acid, and carboethoxy-l-glutamic acid were prepared from the corresponding amine or amino acid and ethylchlorocarbonate by the Schotten–Baumann procedure. The β-hydroxyethylcarbamate was prepared from ethylene glycol and ammonia; the 2-oxazolidone by the method of Homeyer[12]; and the β-aminoethylcarbamate by the method of Ben-Ishai[13]. The carbamyl phosphate was prepared as the lithium salt by the method of Jones *et al.*[14] and this was converted to the sodium salt by passing through an ion exchange column of Amberlite IR-400. The urethane phosphate was prepared by the method of Lapidot and Halmann[15]; while the carboethoxyphosphate, which had not previously been synthesized, was prepared by Halmann and Lapidot by catalytic hydrogenation of dibenzylcarboethoxyphosphate. (It should be noted, however, that this substance proved too unstable for satisfactory characterization, and its use for biological testing might, therefore, have largely involved decomposition products.) The monothiourethane was prepared according to the method of Pinner[16]; the other sulphur analogue, xanthogenamide, according to that of Debus[17].

The urethane was obtained from British Drug Houses Ltd., England; the propyl urethane, butyl amine, diethyl carbonate and ethylene carbonate from Eastman Kodak, Rochester, U.S.A.; the "Miltown" from Lederle Laboratories, New York, U.S.A.; and the croton oil from Magnus, Mabee & Reynard, New York, U.S.A.

Each compound was administered once only, either by mouth or intraperitoneal injection, as aqueous solution in the case of the higher dose ranges, in saline in the case of lower dose ranges, and in medicinal liquid paraffin for compounds that were relatively insoluble in water. The original intention of using molar equivalents had to be abandoned in view of the varying toxicities among the compounds. After an interval of 4 days following the injection, the animals were submitted to twice-weekly applications to the skin of a 5 per cent solution of croton oil in liquid paraffin, for 40 weeks, the applications being limited to an area of about 2 × 2 cm in the dorsal region over the shoulder blades. The croton oil controls were treated the same way, except that they did not receive any preliminary intraperitoneal injection.

The resulting skin tumours were charted at their first appearance, and fortnightly

thereafter; but papillomas that regressed within 2 weeks of their first appearance were not listed in the final records. All the animals were killed at the end of the croton oil paintings, autopsied and the lungs kept for histological examination, to determine more accurately the number of adenomas present in that organ than was possible by visual examination.

RESULTS

Table 1 summarizes the results, both for skin initiating action and lung carcinogenesis, as determined at the end of the experiment, i.e. after completion of eighty applications of croton oil (40 weeks). Since the number of skin tumours after such

TABLE 1. SKIN INITIATING ACTION AND LUNG CARCINOGENESIS BY A SINGLE INTRAPERITONEAL INJECTION (i.p.) OR ORAL ADMINISTRATION (p.o.) OF URETHANE DERIVATIVES

Results after 40 weeks (eighty applications) of subsequent croton oil treatment

Compound	Dose (mg)	Solvent	Route	Number	Skin initiation		Lung carcinogenesis	
					Tumour bearing/ survivors	Average no. tumours per mouse	Tumour bearing/ survivors	Average no. tumours per mouse
Ethyl carbamate (urethane)	25	aq. dist.	p.o.	20	16/18 89%	1·6 ± 0·3	12/18 67%	3·4 ± 1·3
Ethyl carbamate (urethane)	10	saline	i.p.	30	18/30 60%	0·9 ± 0·2	23/26 88%	2·8 ± 0·5
Croton oil (control)	—	—	—	46	11/44 25%	0·4 ± 0·1	2/42 5%	0·05 ± 0·03
Propyl carbamate	25	aq. dist.	p.o.	20	4/14 29%	0·3 ± 0·1	4/14 29%	0·3 ± 0·1
N-Methyl urethane	12·5	aq. dist.	p.o.	20	14/20 70%	1·2 ± 0·4	4/20 20%	0·25 ± 0·1
N-Methyl urethane	11·5	saline	i.p.	30	10/30 33%	0·4 ± 0·1	7/24 29%	0·4 ± 0·1
N:N-Dimethyl urethane	12·5	liq. par.	p.o.	19	6/19 32%	0·4 ± 0·1	0/19 —	—
N-Hydroxy urethane	25	aq. dist.	p.o.	25	19/20 95%	1·5 ± 0·3	9/20 45%	0·75 ± 0·3
N-Hydroxy urethane	25	aq. dist.	i.p.	20	17/18 95%	1·9 ± 0·2	11/18 61%	0·8 ± 0·2
N-Hydroxy urethane	11·8	saline	i.p.	30	6/28 21%	0·2 ± 0·1	5/26 19%	0·3 ± 0·1
N-Hydroxy urethane	5	aq. dist.	i.p.	18	8/18 44%	0·25 ± 0·05	5/18 28%	0·4 ± 0·1
Allyl urethane	12·5	aq. dist.	p.o.	25	15/24 62%	0·8 ± 0·2	0/24 —	—
Allyl urethane	12·7	saline	i.p.	30	6/29 20%	0·25 ± 0·1	2/29 7%	0·07 ± 0·05
β-Hydroxy urethane	25	liq. par.	p.o.	20	6/14 43%	0·4 ± 0·1	1/14 7%	0·07 ± 0·05
β-Amino urethane-HCl	25	aq. dist.	p.o.	10	3/8 37%	0·25 ± 0·1	1/8 12%	0·1 ± 0·1
Oxazolidone	25	aq. dist.	p.o.	17	8/14 57%	0·9 ± 0·3	0/14 —	—
Oxazolidone	10	saline	i.p.	30	10/29 35%	0·35 ± 0·1	0/29 —	—
Monothiourethane	5	saline	i.p.	32	12/30 40%	0·45 ± 0·15	0/30 —	—
Xanthogenamide	2	saline	i.p.	26	4/25 16%	0·15 ± 0·05	4/23 17%	0·2 ± 0·1
Butylamide	20	saline	i.p.	29	9/29 31%	0·55 ± 0·15	3/24 12%	0·2 ± 0·1
Diethyl carbonate	12·5	aq. dist.	p.o.	20	11/15 73%	1·1 ± 0·2	1/15 7%	0·05 ± 0·05
Diethyl carbonate	11·4	saline	i.p.	30	12/30 40%	0·4 ± 0·1	0/30 —	—
"Miltown"	7	saline	i.p.	20	6/20 30%	0·3 ± 0·1	0/20 —	—
Carbamyl phosphate	30	aq. dist.	p.o.	22	11/18 61%	0·85 ± 0·2	0/18 —	—
Carbamyl phosphate	30	aq. dist.	i.p.	20	10/18 55%	0·55 ± 0·2	1/18 5%	0·05 ± 0·05
Potassium cyanate	2·5	saline	i.p.	20	7/15 47%	0·6 ± 0·2	0/15 —	—
Carboethoxyphosphate	5	saline	i.p.	26	4/9 44%	0·4 ± 0·2	4/9 44%	0·9 ± 0·5
Urethane phosphate	5	saline	i.p.	33	8/24 33%	0·4 ± 0·1	2/16 12%	0·1 ± 0·08
Carboethoxyaspartic acid	5	saline	i.p.	30	11/24 46%	0·55 ± 0·15	1/24 4%	0·04 ± 0·04
Carboethoxyglutamic acid	5	saline	i.p.	40	11/40 28%	0·3 ± 0·1	0/40 —	—
Carboethoxyalanine	5	saline	i.p.	10	4/10 40%	0·3 ± 0·1	0/10 —	—
Carboethoxyalanine	1	saline	i.p.	30	10/30 33%	0·3 ± 0·1	1/28 4%	0·04 ± 0·03
Carboethoxyglycine	1	saline	i.p.	30	5/18 28%	0·3 ± 0·1	1/18 6%	0·06 ± 0·04

prolonged treatment with croton oil is relatively high in the controls, due to background carcinogenicity of the croton oil itself, the tumour yields in the various groups after only forty applications (20 weeks) are presented separately in Table 2. These data naturally refer to skin tumours only, since lung adenomas cannot be recognized during life. The background carcinogenicity of croton oil, at the twentieth week, being relatively low, makes it easier for the significance of differences between experimental and control groups, in the case of skin tumours, to be assessed at that stage. Since the varying dosage prevents quantitative comparisons of intensities of action, the results are finally summarized in Tables 3–6 on a semiquantitative basis, i.e. by plus and

minus signs, indicating overall differences without claiming accuracy about minor differences.

TABLE 2. SKIN INITIATING ACTION BY A SINGLE INTRAPERITONEAL INJECTION (i.p.) OR ORAL ADMINISTRATION (p.o.) OF URETHANE DERIVATIVES

Results after 20 weeks (forty applications) of subsequent croton oil treatment

Compound	Dose (mg	Solvent	Route	No.	Tumour bearing/ survivors		Average no. tumours per mouse
Ethyl carbamate (urethane)	25	aq. dist.	p.o.	20	16/18	89%	1·5 ± 0·2
Ethyl carbamate (urethane)	10	saline	i.p.	30	14/30	47%	0·6 ± 0·1
Croton oil (control)	—	—	—	46	3/45	7%	0·07 ± 0·05
Propyl carbamate	25	aq. dist.	p.o.	20	3/15	20%	0·2 ± 0·1
N-Methyl urethane	12·5	aq. dist.	p.o.	20	6/20	30%	0·3 ± 0·08
N-Methyl urethane	11·5	saline	i.p.	30	4/30	13%	0·17 ± 0·08
N:*N*-Dimethyl urethane	12·5	liq. par.	p.o.	19	0·19	0%	—
N-Hydroxy urethane	25	aq. dist.	p.o.	25	12/25	48%	0·7 ± 0·2
N-Hydroxy urethane	25	aq. dist.	i.p.	20	14/20	70%	10· ± 0·2
N-Hydroxy urethane	11·8	saline	i.p.	30	3/29	10%	0·1 ± 0·05
N-Hydroxy urethane	5	aq. dist.	i.p.	18	4/18	22%	0·3 ± 0·1
Allyl urethane	12·5	aq. dist.	p.o.	25	4/24	17%	0·2 ± 0·05
Allyl urethane	12·7	saline	i.p.	30	3/29	10%	0·1 ± 0·05
β-Hydroxy urethane	25	liq. par.	p.o.	20	2/19	10%	0·2 ± 0·1
β-Aminourethane-HCl	25	aq. dist.	p.o.	10	1/9	11%	0·1 ± 0·01
Oxazolidone	25	aq. dist.	p.o.	17	1/16	6%	0·05 ± 0·05
Oxazolidone	10	saline	i.p.	30	4/29	14%	0·14 ± 0·05
Monothiourethane	5	saline	i.p.	32	4/30	13%	0·13 ± 0·07
Xanthogenamide	2	saline	i.p.	26	3/25	12%	0·12 ± 0·07
Butylamide	20	saline	i.p.	29	9/29	31%	0·3 ± 0·1
Diethyl carbonate	12·5	aq. dist.	p.o.	20	3/17	18%	0·2 ± 0·1
Diethyl carbonate	11·4	saline	i.p.	30	4/30	13%	0·13 ± 0·05
"Miltown"	7	saline	i.p.	20	1/20	5%	0·05 ± 0·05
Carbamyl phosphate	30	aq. dist.	p.o.	22	2/19	11%	0·1 ± 0·01
Carbamyl phosphate	30	aq. dist.	i.p.	20	2/18	11%	0·1 ± 0·01
Potassium cyanate	2·5	saline	i.p.	20	2/20	10%	0·15 ± 0·1
Carboethoxy phosphate	5	saline	i.p.	26	3/21	14%	0·15 ± 0·1
Urethane phosphate	5	saline	i.p.	33	3/26	12%	0·12 ± 0·05
Carboethoxyaspartic acid	5	saline	i.p.	30	2/24	8%	0·08 ± 0·05
Carboethoxyglutamic acid	5	saline	i.p.	40	3/40	7%	0·08 ± 0·05
Carboethoxyalanine	5	saline	i.p.	10	0/10	0%	—
Carboethoxyalanine	1	saline	i.p.	30	3/30	10%	0·1 ± 0·05
Carboethoxyglycine	1	saline	i.p.	30	1/21	5%	0·05 ± 0·05

DISCUSSION

It was hoped that one or other of the tested derivatives would prove more potent than urethane itself and thus provide a lead as to the nature of the postulated effective metabolite. This hope was not realized. On the contrary, the few compounds that did elicit a positive response, whether for skin initiation or lung carcinogenesis, were decidedly weaker in action than urethane.

The question then arose whether the complete lack of activity of some of the compounds might throw light on the problem of urethane metabolism in a *negative sense*, i.e. by enabling one to eliminate certain metabolic mechanisms that might otherwise have been considered possible on *a priori* grounds.

On the assumption that the effective metabolite is an intermediate product in the hydrolytic breakdown of urethane, one might suspect either the carbamyl or the carboethoxy group to be the essential portion of the urethane molecule involved in

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the biological action, reacting either directly with constituents of the living cell, or indirectly in the form of carbamyl or carboethoxy derivatives.

Carbamyl portion

$$\overbrace{NH_2—CO}—O—C_2H_5$$

$$NH_2—\underbrace{CO—O—C_2H_5}$$

Carboethoxy portion

Direct action of the free, unstable, radical could not be determined by the experimental methods discussed here; indirect action by derivatives could, however, be examined by testing the suspected complexes, synthetically produced, for skin initiating action and lung carcinogenesis. Two classes of such complexes were examined: (*a*) phosphorylated derivatives (Table 5); and (*b*) complexes of urethane with keto-acids, which may also be considered as carboethoxy derivatives of amino acids (Table 6). It was found, however, that none of the compounds in either series had more than borderline activity for skin initiation or lung carcinogenesis. Most of them were, in fact, completely inactive.

Table 3. Modification of the carbamyl portion of the urethane molecule

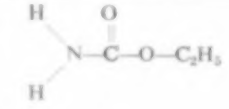
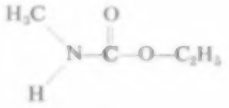
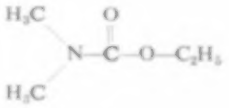
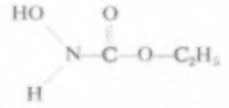
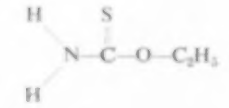
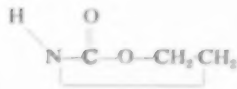
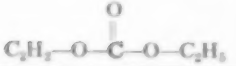
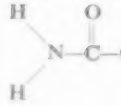
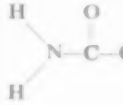
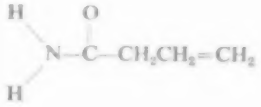
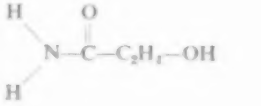
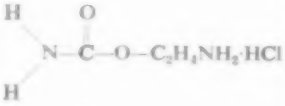
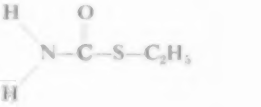
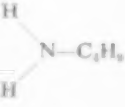
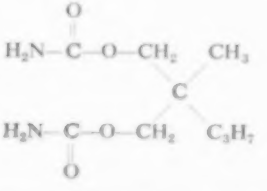
Compound	Formula	Initiating action on skin	Carcinogenic action on the lungs
Ethylcarbamate (urethane)		+ + + + +	+ + + + +
N-Methylethylcarbamate		+ +	+ +
N:*N*-Dimethylethylcarbamate		—	—
N-Hydroxyethylcarbamate		+ + +	+ +
Xanthogenamide		(?) ±	—
Oxazolidone		+	—
Diethylcarbonate		+	—

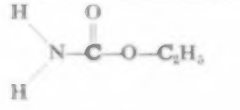
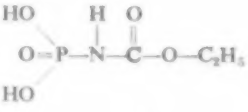
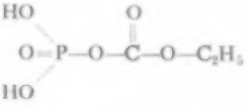
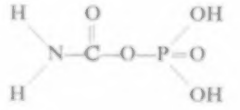
TABLE 4. MODIFICATION OF THE CARBOETHOXY PORTION OF THE URETHANE MOLECULE

Compound	Formula	Initiating action on skin	Carcinogenic action on the lungs
Ethylcarbamate (urethane)		+ + + + +	+ + + + +
Propylcarbamate		+	+
Allylcarbamate		+	—
β-Hydroxyethylcarbamate		(?) ±	—
β-Aminoethylcarbamate HCl		—	—
Monothiourethane		(?) ±	—
Butylamide		+	—
2-Methyl-2-*n*-propyl 1:3-propane diol dicarbamate (Miltown)		—	—

The fact that, among the phosphorylated derivatives some borderline activity (initiating action on the skin) was noted in the case of carbamyl phosphate, was surprising, since this compound is known to be a normal constituent of the body. One possible explanation of the result—that the preparation might have contained some urethane as contaminant—was rigorously excluded by chemical tests. The original

experiment was then repeated, and slight initiating action noted once again (see Table 5). These results should, nevertheless, be accepted with reservations, being based on narrow differences between the experimental and control values. Meanwhile, the search was carried a stage further, on the basis of the observation[18] that carbamyl phosphate liberates cyanate on decomposition under certain conditions. The latter was, therefore, also tested for skin initiation and lung carcinogenesis, but found to be inactive. Carboethoxyphosphate also appeared to be active, at least for lung carcinogenesis, though the results were not decisive in view of the poor survival of the treated animals due to the toxicity of the material.

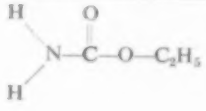
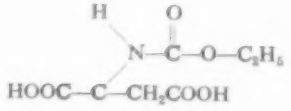
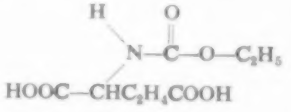
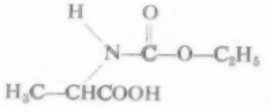
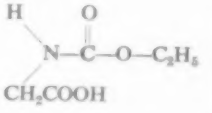
TABLE 5. PHOSPHORYLATED DERIVATIVES

Compound	Formula	Initiating action on skin	Carcinogenic action on the lungs
Ethylcarbamate (urethane)		+ + + + +	+ + + + +
Urethane phosphate		(?) ±	(?) ±
Carboethoxyphosphate		−	+
Carbamyl phosphate		(?) ±	−
Potassium cyanate*	KCNO	(?) ±	−

* Decomposition product of carbamyl phosphate.

Another approach was to test "artificial" derivatives of urethane (i.e. those not suspected of appearing metabolically in the body), with substituents in the carbamyl and in the carboethoxy portions, respectively, of the urethane molecule. If such derivatives invariably failed to show activity when the substituent was at one end of the molecule, but retained activity when the substituent was at the other end, the argument could be considered that the group in which "non-biological" substitution caused repression was the one essential for biological activity. From this point of view, the results (see Tables 3 and 4) were not conclusive, since considerable repression occurred in both series. But the fact that diethyl carbonate had any activity at all suggests that the presence of the amino (i.e. the carbamyl) group is not essential, and therefore, by implication, that the carboethoxy group might be the important group.

TABLE 6. COMPLEXES OF KETOACIDS WITH URETHANE, REPRESENTING AMINO ACID DERIVATIVES OF THE CARBOETHOXY GROUP

Compound	Formula	Initiating action on skin	Carcinogenic action on the lungs
Ethylcarbamate (urethane)		+ + + + +	+ + + + +
Carboethoxyaspartic acid		–	–
Carboethoxyglutamic acid		–	–
Carboethoxyalanine		–	–
Carboethoxyglycine		–	–

A point of particular interest was that both sulphur analogues of urethane were virtually inactive. This is in keeping with the idea that the carboethoxy portion of the urethane molecule must remain intact for effective biological action. If the carbamyl were the critical portion, one would have expected only one of the two sulphur analogues to be inactive, namely the monothiourethane, i.e. with the sulphur substitution in the carbamyl portion. When the sulphur substitution is in the carbonyl group, both portions are naturally implicated.

In a study of the carcinogenicity of a series of esters of carbamic acid, with respect to lung carcinogenesis, Larsen[19] found that the high activity, in the case of the ethyl derivative, rapidly falls off when this group is replaced by *iso*propyl, and more so with the *n*-propyl, while the methyl, *n*-butyl and *iso*amyl derivatives are inactive. This indicated not only that the structural integrity of urethane is more critical for lung carcinogenesis than for its anaesthetic and other biological properties, but also suggested that the carboethoxy group is particularly important. On the other hand significant activity was also observed with tribromoethyl carbamate.

In a subsequent investigation by Larsen[20], a similar study was made with *N*-alkylated derivatives, i.e. with substitution at the carbamyl end of the urethane molecule. While activity was reduced in many cases, the specificity was not as critical as in the case of carbamic acid esters. Thus, fairly high activity was found in the case of *iso*propylurethane and methylene and ethylene diurethane, and lower activities with many of

the other derivatives. These results add further weight to the belief that carcinogenic activity is dependent on the carboethoxy rather than the carbamyl group in the urethane molecule being intact.

Reference should also be made to a recent report[21] that *iso*propyl-N-phenyl carbamate and *iso*propyl-N-(3-chlorophenyl) carbamate are weak but definite initiators for mouse skin carcinogenesis.

Finally, there is the possibility that the effective metabolite might arise not as an intermediate product of the *hydrolytic* breakdown of urethane, but by some *oxidative* mechanism, through a side reaction. Though such a metabolic mechanism has never been demonstrated in animals, some evidence for it was suggested by Isenberg *et al.*[22], though subsequent work by that group cast doubts on their earlier interpretations.[23] It should be noted, in this connexion, that in our investigations, *N*-hydroxyurethane had a decided action, both as skin initiator and lung carcinogen, though with a lower potency than that of urethane. However, hydroxyethyl urethane was inactive. (In the preliminary report,[1] the latter compound was also listed as a weak initiator, but more complete analysis convinced us that the observed differences between it and the control were not statistically significant.)

While these observations may serve as pointers for further study, they do not permit any overall conclusion on the essential problem that prompted the present investigation.

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INCREASE OF ACID PHOSPHATASE ACTIVITY IN MOUSE LIVER AFTER DEXTRAN STORAGE

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Abstract—Dextran solutions were injected into the peritoneal cavity of mice and sections of liver tissue were studied histochemically. A rise in acid phosphatase activity was found in liver parenchymal cells correlated with storage of dextran granules in the same cells. In homogenates of these livers the acid phosphatase activity was found to be approximately doubled.

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THE microscopical histochemical methods for enzymes such as those for alkaline and acid phosphatase[1] can reveal in a semiquantitative way the distribution pattern of these enzymes in the different types of cells and in cells in varying functional states present in tissues. The distribution patterns of these phosphatases were studied[2] in sections of human tissues removed surgically or at autopsies, with the aim of finding relations between the enzyme patterns of normal and pathological functional states of cells.

With regard to acid phosphatase (AP) distribution, one of us[3] was struck by the high acid phosphatase activity consistently seen in cells of the macrophage type. A high AP activity, evidenced by a dense precipitate of brown–black PbS, was found for instance in the macrophages present in chronically inflamed lungs and in macrophages around a degenerating corpus luteum. The possible existence of a relation between storage in cells and a high AP activity seemed further strengthened by the high AP activity found in the reticuloendothelial cells, such as those in liver and spleen, which are known for their capacity to store many materials of microscopical and macromolecular dimensions[4] in their cytoplasm. The cells of the reticuloendothelial system can be brought into a state of maximal storage by injecting animals with cell-foreign substances of several kinds. A histochemical and quantitative biochemical study of the relation between AP activity and the storage phenomenon seemed therefore possible.

Several substances, most of them coloured, such as indian ink particles and trypan blue, are known to be stored in reticuloendothelial cells. For the present investigation the plasma-substitute dextran was chosen because it is a colourless substance not disturbing the observations of the brown–black PbS in the sections after performing the Gomori method for AP localization.

Storage of dextran has been demonstrated histochemically by Mowry and Millican[5]. After intravenous or intraperitoneal administration of dextran, their alcoholic periodic acid Schiff method reveals its presence as granules of microscopic dimensions in cells of several organs.

We injected relatively high doses of dextran intraperitoneally into mice and observed, in agreement with the results of Mowry and Millican, that in the livers of

those animals dextran granules were present in high quantity in the Kupffer cells, but that at these high doses, these granules were also found in the parenchymal cells. In sections of the same material stained histochemically with the Gomori AP technique, a possible increase in AP activity in the Kupffer cells could not be established by visual observation, since these cells already reacted intensely in the livers of control animals. It was found, however, that the liver parenchymal cells gave a strong reaction for AP in dextran-treated mice but were only weakly positive in the untreated animals. This histochemical observation already provided some evidence for the postulated relation between AP and storage. The present paper deals predominantly with the results of the quantitative determination of AP activity in homogenates of mice liver from dextran-injected animals compared with those from control animals. It was found that dextran injection resulted in a significant rise in AP activity in livers of the animals thus treated compared with livers of control animals.

MATERIALS AND METHODS

Experimental animals. Young Swiss mice of own stock of both sex were used, weighing about 20 g, aged about 6 weeks.

Substrate. Glycerophosphate (sodium β-glycerophosphate, $Na_2C_3H_7O_6P{\cdot}5H_2O$, Merck) was used as substrate both in the histochemical and biochemical experiments. This product showed the following characteristics: loss of weight after drying at 135°C, 29·38 per cent (calc. 29·43 per cent); loss of weight on ignition 56·50 per cent (calc. 56·57 per cent); percentage inorganic P from total P: 0·05 per cent; the product contained 23·9 per cent of the α-isomer (determined according to Toal and Phillips[6]). Melting range 91°–94·5°C (corrected). For the pure β-isomer a melting range of 100–102°C is reported by Ashby[7].

Dextrans. Dextran with an average molecular weight of 75,000 was purchased from Poviet Co., Amsterdam (licensed by Pharmacia, Stockholm) and can be obtained as "Macrodex", a 6 per cent sterile solution in 0·9 per cent NaCl for clinical use. Dextran with average molecular weight of 160,000 was a gift from Poviet Co. and was obtained as a white powder from which a 6 per cent sterile solution in 0·9 per cent NaCl was prepared.

Injection scheme. Seven groups of animals were examined. The animals were sacrificed on the tenth day after the day of the first injection and AP activity determined in homogenates of the livers. A control group (I) received no treatment. A second group (II) was made to fast for 18 hr before being killed, ample supply of drinking water remaining available. Group (III) were given daily intraperitoneal injection of 1 ml 0·9 per cent NaCl during nine days and were made to fast 18 hr before being sacrificed at the tenth day. Group (IV) were given a daily injection of the 75,000 dextran during five days, received no treatment during four days and were made to fast 18 hr. Group (V) were given nine daily injections of 1 ml of the same dextran solution as group (IV) and were made to fast for 18 hr. Groups (VI) and (VII) received the same treatment as groups (IV) and (V), respectively, except that the dextran sample had an average molecular weight of 160,000.

The injection scheme for each animal was chosen in such a way that one animal of each group was sacrificed on the same day. Systematic variations possibly present in enzyme determinations or unknown change in the conditions of the animals from one day to another were thus eliminated.

The animals were sacrificed by decapitation and bled. Through light pressure in the abdominal region the livers were more or less freed from blood.

Histochemistry. Pieces of liver tissue (ca. 5 × 5 mm) were fixed in cold acetone, embedded in paraffin, sectioned and stained for acid phosphatase according to the method of Gomori[1] as modified by Goetsch and Reynolds[8], and stained for dextran according to the method of Mowry and Millican[5].

Preparation of homogenates. Small quantities of tissue (ca. 200 mg), taken from different parts of the liver, were rapidly weighed on a torsion balance. The tissue was homogenized during 1½ min in a ground glass Potter–Elvejhem homogenizer in about 20 ml distilled water in a cold room (ca. 0°C). After homogenizing, distilled water was added to bring the tissue concentration at 100 mg tissue/20 ml water. To 10 parts of this solution were added 1 part of a 1 per cent solution of Triton-X-100 (a gift from Rohm and Haas Co., Philadelphia, U.S.A.) to achieve a maximal solubilization and availability of the enzyme for interaction with its substrate.[9] The homogenates to which Triton-X-100 had been added, were allowed to stand at room temperature for 15 min.

Enzyme activity estimation. Acid phosphatase activity was determined by incubating 2 ml of the homogenate with 10 ml of a 0·2 M acetate buffer pH 4·8 and 2 ml substrate solution containing 50 mg/ml sodium β-glycerophosphate at 37°C for 1 hr. Inorganic phosphate was determined in 2 ml aliquots of the incubating mixture before and after the incubation period, using the method of Lindberg and Ernster[10]. Triton-X-100 in the concentration used was found not to disturb this determination.

Aliquots of the homogenates were analysed for total nitrogen by the Kjeldahl method.[11]

RESULTS

Effect of dextran injections on AP distribution in liver cells

In the livers of the animals, not injected with dextran, AP was predominantly present in the cytoplasm of the Kupffer cells; in the liver parenchymal cells only small quantities of AP could be demonstrated. In the livers of animals to which dextran had been administered dextran granules were found both in the Kupffer cells and in the liver parenchymal cells.

Thus the AP distribution in these groups of animals differed from the control groups in a considerable higher activity of AP in the parenchymal cells. Whether the activity in the Kupffer cells had changed remained uncertain.

Acid phosphatase activity in homogenates

The results of the AP activity estimations are assembled in Table 1. The AP activity in each of the dextran groups (V), (VI) and (VII), the day after the injection period ended was found to be considerably higher than in the control groups (I), (II) and (III). According to Fisher's "*t* test"[12] the "*P*" values were considerably lower than 0·05. The group injected with dextran 75,000 during 5 days only, showed a mean enzyme activity higher than the control groups. The difference however was not significant ($P > 0{\cdot}05$).

DISCUSSION

The experimental results show that a considerable increase in the AP activity of mouse liver homogenates occurs together with storage of dextran in this organ.

TABLE 1. ACID PHOSPHATASE ACTIVITY IN LIVER HOMOGENATES OF DEXTRAN-INJECTED AND CONTROL GROUPS

Group	Injection period	Average body weight* (g)	Acid phosphatase activity	
			μg P/100 mg wet liver†	μg P/mg N‡
(I) controls (not fasted)	—	23 ± 3	269 ± 41	78 ± 12
(II) fasted	—	21 ± 4	319 ± 48	95 ± 14
(III) 0·9% NaCl	9 days	21 ± 2	339 ± 18	98 ± 8
(IV) dextran, mol. w. ca. 75,000	5 days	20 ± 4	398 ± 83	126 ± 27
(V) dextran 75,000	9 days	22 ± 4	531 ± 104	176 ± 35
(VI) dextran, mol. w. ca. 160,000	5 days	22 ± 3	493 ± 91	153 ± 29
(VII) dextran, 160,000	9 days	20 ± 3	549 ± 13	169 ± 9

* Weight at the end of the experiment, each group consisted of five animals (±s.d.).
† Activity expressed in μg P produced per 100 mg wet liver tissue per hr (± s.d.).
‡ Activity expressed in μg P produced per mg total nitrogen per hr (± s.d.).

A similar result had been obtained several times in preliminary experiments not reported here. In these experiments the mean value of AP activity in the livers of control animals was found to increase with the age of the animals. The impression was gained that the older animals the percentage increase in AP activity after dextran injection is lower. However, in every experiment carried out so far the increase found was statistically significant. The effect of dextran injection on AP activity in mouse liver can therefore be considered established. An increase of the magnitude observed cannot be explained by changes in the glycogen level, since the difference in AP activity between fasting and non-fasting animals is much smaller than the increase seen in the dextran groups.

The increase can neither be due to an unspecific "stress" effect caused by the injections as such, as is demonstrated by the values found in the saline injected control group. The average molecular weight of the dextran used as well as the length of the injection period has some influence on the results. The dextran with an average molecular weight of 75,000 is unable to give a strong increase when injected for 5 days only.

The histochemical findings show that much of the increase of AP activity is localized in the parenchymal cells of the liver. It was not possible to establish whether a similar increase had occurred in the Kupffer cells. A separate evaluation of the effects of dextran injections under the conditions of our experiment on the activity and number of Kupffer cells remains desirable. The experiments were planned to give biochemical support for the histochemical finding of a high AP activity of cells when they store metabolically inert substances in their cytoplasm. However, dextran, though apparently of such inertness that cells have to store it for long times without being able to metabolize it, has been found not to be completely metabolically inert. It is known to be metabolized, though very slowly in the animal organism especially in the liver.[13, 14] So far no indication of the nature of enzymes involved in dextran metabolism seems have been gained, but regarding the metabolism of its monomer glucose, no indication for a possible role of acid phosphatase can be found. It is therefore most likely that our results indicate the existence of a special relationship between acid phosphatase activity and the storage of cell foreign substances. It should be emphasized that the

storage of substances is preceded by the uptake of the particles (phagocytosis or pinocytosis) and followed by a non-metabolic elimination. On basis of the long injection period necessary (groups (IV) and (V)) there seems some indication that AP is not directly correlated with the uptake itself. The phenomena of phagocytosis or pinocytosis followed by storage in different types of cells such as rat liver, rat kidney and Hela cells have been related to small cytoplasmatic granules,[15] called dense bodies, microbodies, Straus's droplets, peribiliary bodies, microkinetospheres, etc.[16] These type of granules as far as they have been studied cytochemically have been found to be rich in acid phosphatase.[17, 18]

The present finding demonstrates that AP activity in mouse liver can be influenced experimentally. This opens a possibility to approach the relation between AP activity and these granules in an object suitable for further biochemical, morphological and cytochemical study.

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METABOLISM OF THIOPYRIMIDINE DERIVATIVES: THIAMYLAL, THIOPENTAL AND THIOURACIL*

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Abstract—The metabolic fate of three pharmacologically significant thiopyrimidines has been investigated. Incubation of thiamylal, 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid, with minced rat liver resulted in the formation of a metabolite which was isolated and identified as secobarbital. The urine of rats receiving thiouracil, 2-mercapto-4-hydroxypyrimidine, was examined by ion exchange and paper chromatography and revealed significant amounts of uracil. Thiouracil, when incubated with a mince or homogenate of rat liver, was metabolized at the rate of 280–350 μg/g of tissue per 3 hr. Significant amounts of ammonia were formed during this time. The supernatant fluid from such incubated mixtures contained a metabolite which was identified chromatographically as β-alanine. Significant quantities of uracil also were shown to be present. These findings suggest a metabolic pathway in the rat for thiouracil which involves first, desulphuration with the formation of uracil, followed by cleavage of the thiopyrimidine ring and formation of β-alanine, ammonia and carbon dioxide. Rats receiving thiouracil-2-C^{14} were found to excrete about 80 per cent of the radioactivity in the urine during the first 24 hr. Approximately 1·9 per cent of the excreted radioactivity was extractable with chloroform and 6·2 per cent was removed by extraction with ethylene dichloride. Data are presented which indicate that approximately 16·5 per cent of the remaining radioactivity is uracil-2-C^{14}.

Thiopental, 5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid, was found to be metabolized by the microsomal fraction of rat liver. Reduced triphosphopyridine nucleotide, magnesium ion and oxygen were essential for a maximum rate of metabolism. Pentobarbital was isolated from this enzymic system by countercurrent distribution and sublimation and characterized by ultra-violet and infra-red spectral analysis. Metabolic conversion was not greatly inhibited by cyanide ion but was markedly inhibited by β-diethylaminoethyl diphenylpropylacetate (SKF 525-A). Thiopental was metabolized at an average rate of 0·3 μmole/3 hr by the enzymic activity present in the quantity of microsomes obtained from 1 g of liver. Rates of metabolism of thiopental by microsomal preparations of liver from the guinea pig and pigeon were approximately one-half and one-third, respectively, those of the dog, rabbit and mouse, all of which exhibited about the same activity and two-thirds that of rat liver.

THE observation by Winters *et al.*[1] that a significant amount of thiopental is converted to its oxygen analogue *in vitro* by preparations of rat liver demonstrated that desulphuration is an important metabolic alteration in the biotransformation of a thiobarbiturate. The studies reported herein were undertaken to determine whether or not

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desulphuration represents a metabolic mechanism common to thiopyrimidines other than thiopental. Thiamylal, 5-allyl-5-(1-methylbutyl)-thiobarbituric acid, another thiobarbiturate and thiouracil, one of the simplest members of the thiopyrimidine group, were selected for this purpose. Information on the intracellular distribution and characteristics of the enzymic system involved in the desulphuration of thiopyrimidines has also been obtained.

METHODS AND RESULTS

Thiamylal studies

Mature female rats of the Holtzman strain, weighing between 300 and 350 g, were killed by a blow on the head then decapitated. The livers were removed and minced in a previously chilled Seevers–Shideman[2] mincing unit. The minced tissue was collected in a tared, iced beaker, weighed and a sufficient amount of chilled, isotonic, calcium-free, Krebs–Ringer phosphate buffer was added to make an 11.1 per cent mixture of mince in the buffer. One ml of a 0·1 per cent solution of thiamylal sodium was added to each 9 ml of tissue mixture which previously had been pipetted into chilled 50 ml beakers. After the addition of the drug, each beaker was shaken gently for a period of from 10 to 15 sec. to ensure a uniform distribution of drug in the incubational mixture. An aliquot of 1 ml was immediately removed from each beaker and analyzed for thiamylal concentration by the method described by Brodie *et al.*[3] for the estimation of thiopental. The beakers were placed in a Dubnoff metabolic shaking incubator and incubated in an atmosphere of oxygen at a shaking rate of 120 oscillations per min for 3 hr at 37–38 °C. After this time, aliquots were again removed from the beakers previously sampled and analyzed for their thiamylal concentration.

When thiamylal was incubated under these conditions, an average of 300 μg (s.e. = $\pm$37 μg) of the added drug disappeared in a period of 3 hr. Winters *et al.*[1] found that when comparable concentrations of thiopental were employed, approximately 42 per cent of the added drug disappeared in the same period of time. The rate in their experiments was about 420 μg/g of tissue in 3 hr.

Extraction procedure. Following the removal of the aliquots at the termination of the incubational period, the preparations were pooled and acidified to a pH of approximately 3. The acidified mixture was extracted with reagent grade diethyl ether in a continuous liquid–liquid extractor for a period of from 24 to 48 hr. The ether extract was evaporated to dryness, the residue was dissolved in 100 ml of chloroform and shaken with an equal volume of 0·2 M borate buffer of pH 8·8 for 30 min. The buffer phase was discarded and fresh buffer was added. The partitioning procedure was repeated twice. In this manner a significant amount of thiamylal (approximately 18 per cent) was removed from the chloroform without extracting significant quantities of its suspected metabolite, secobarbital. The chloroform was removed by evaporation and the residue subjected to countercurrent distribution.

Countercurrent distribution. The above residue was dissolved in 10 ml of chloroform which previously had been saturated with borate buffer, pH 10·5*. A 14-plate countercurrent distribution was then performed using a volume of borate buffer equal to that of the chloroform as the stationary phase. The procedure was carried out in glass stoppered, 34 ml reaction vessels or in 250 ml separatory funnels. The ultra-violet

* The buffer solution was prepared as follows: Solution *A*: Na_2CO_3, 5·3 g/l. Solution *B*: $Na_2B_4O_7$, 19·1 g/l. Solutions *A* and *B* were mixed to provide a buffer of pH 10·5.

absorption spectrum of the buffer phase from each vessel in the countercurrent train was determined using buffer saturated with chloroform as the blank.

In Fig. 1 are illustrated the results of a representative experiment. Five such experiments were performed. It was found that the material in tubes 0 to 5 exhibited an absorption peak at 240 mμ. The aqueous phases of tubes 2 to 12 displayed an absorption peak at 305 mμ. The calculated *K*-value (ratio of the concentration in lower to upper phase) of the peak at 240 mμ was 0·182, while the other peak displayed a *K*-value of 1·03. The latter *K* is identical with the experimentally determined coefficient for thiamylal in this system. A similarly determined *K*-value for secobarbital was 0·188.

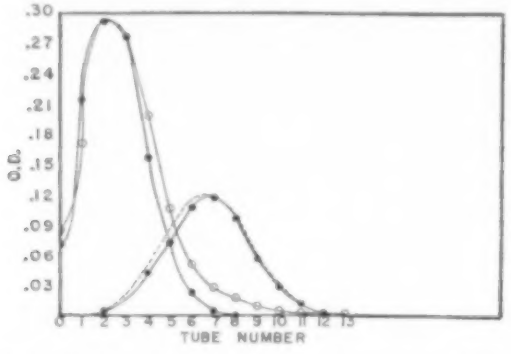

FIG. 1. Countercurrent distribution of material in the ether extract of an acidified incubational mixture. The optical densities (buffer phase) at 240 mμ and 305 mμ of each tube are illustrated.
○ O.D. at 240 mμ; ----- O.D. at 305 mμ; ● calculated

Isolation and characterization of the metabolite. The presence of this material was indicated by the results obtained in the above experiment, i.e. the presence of a material exhibiting an absorption peak at 240 mμ. To obtain a sufficiently large quantity of metabolite for purposes of isolation and identification, a large scale experiment of the type used in the above studies was performed. Thiamylal (100 mg) was added to 100 g of a mince of rat liver suspended in 900 ml of ice cold, Krebs–Ringer phosphate buffer at pH 7·4 and shaken vigorously for 1 min to ensure a uniform mixture. Equal volumes of this mixture were placed in each of four 1 l. Erlenmeyer flasks. The concentration of thiamylal was estimated in duplicate on aliquots obtained from each flask. The flasks were oxygenated for a period of 5 min, stoppered and placed on a rotary shaker in a room maintained at a constant temperature of 38 °C. During the 3 hr incubational period, the mixtures were oxygenated every 20 min for a period of 5 min. Immediately following the incubation period, aliquots were removed from the flasks and analyzed for their content of thiamylal. The pooled mixtures were acidified and extracted for a period of 48 hr in a continuous extractor with diethyl ether. The ether extractable material was prepared for countercurrent distribution in the manner previously described. This study revealed the presence of the metabolite in highest concentration in tubes 0 to 3. These tubes were acidified to a pH of 4, shaken for a period of 1 hr, then centrifuged at 1500 rev/min for 15 min. The organic phases were removed and combined. The chloroform was evaporated to dryness under a stream of cold air and the residue was dried overnight

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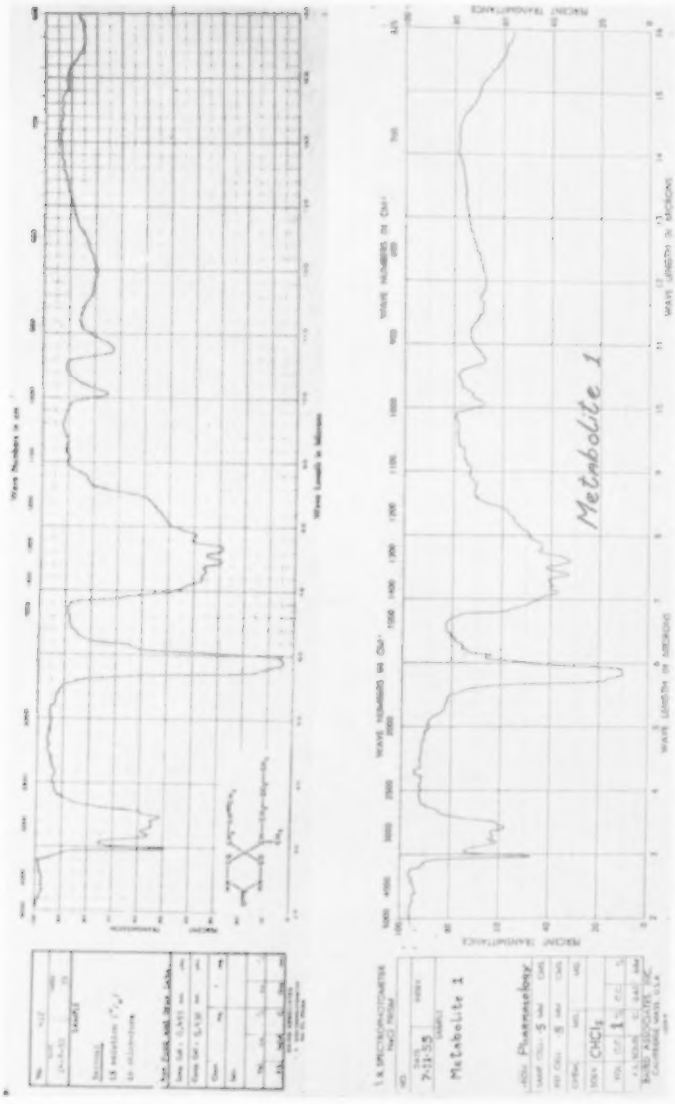

FIG. 2. Infra-red absorption spectra of secobarbital and the metabolite of thiamylal.

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in a vacuum desiccator. Following this the material was dissolved in acetone, pipetted into a 5 ml sublimation tube, and the acetone evaporated by placing the lower half of the tube in a boiling water bath. The residue was sublimed under vacuum (0·03–0·04 mm Hg). The sublimates which appeared were placed in a vacuum desiccator for 2 weeks during which time a white crystalline material formed.

The assumption that the metabolite was an oxybarbiturate rather than a thio-derivative was based on its characteristic ultra-violet absorption spectrum. (In an alkaline medium the barbituric acid derivatives absorb maximally in the vicinity of 240 mμ. In the case of the thiobarbituric acids this maximum disappears and is replaced by one at 305 mμ.) Infra-red spectroscopy* was employed as one aid in identifying the biotransformation product of thiamylal. The details of the preparation of the metabolite for analysis were as nearly as possible the same as those previously employed when an infra-red absorption spectrum of authentic secobarbital was determined. These results are illustrated in Fig. 2. The absorption spectrum for the thio-derivative differs from those of the metabolite and secobarbital in exhibiting significant absorption maxima at approximately 4·2 and 8·9 μ. The melting point† of the metabolite was 84°C. Authentic secobarbital melted at 82·8–83·0°C and a mixed melting point of this material and the metabolite was determined to be 83·5°C. Further evidence for the identity of the metabolite derives from elemental analysis‡ which provided the following results: authentic secobarbital, C, 60·48 per cent; H, 7·61 per cent; N, 11·75 per cent; metabolite, C, 60·97 per cent; H, 7·56 per cent; N, 11·91 per cent.

Thiouracil studies

In vivo studies. Eight adult female albino rats received, 20 ml/kg of water orally 20–30 min prior to the intraperitoneal injection of thiouracil (100 mg/kg). Two animals were placed in each of four metabolism cages, allowed free access to food and water, and urine was collected for from 24 to 36 hr. Following filtration through cheese-cloth, the combined urines were washed by shaking for 30 min with 2 vols. of petroleum ether (b.p. 40–45°C). After centrifuging the mixture at 3000 rev/min for 15 min, the organic phase was discarded. The urine was again washed with petroleum ether, then subjected to successive washings with equal volumes of chloroform or carbon tetrachloride and ethylene dichloride. The aqueous solution remaining after such treatment was concentrated *in vacuo* at room temperature to a volume of from 10 to 15 ml.

Column chromatography. The preparation of the Dowex-1-formate resin was similar to that described by Winters *et al.*[1]. A 5 ml sample of the concentrated, washed urine, prepared as described previously was placed on the resin bed and the sides of the glass column were washed with from 20 to 25 ml of water. Utilizing the method of gradient elution described by Busch, Hurlbert and Potter[4], the column was eluted with gradually increasing concentrations of ammonium or sodium formate. The formate solution (1 M), under continuous pressure, was forced into a mixing

* An infra-red absorption spectrum of the metabolite, prepared as a 1 per cent solution in 0·5 ml chloroform, was obtained, using a Baird-Atomic NaCl Prism Infra-red Spectrophotometer.

† Melting points were determined with the aid of a Fisher–Johns melting point apparatus. Mixed melting points were carried out with a sample of 5-(1-methylbutyl)-5-allyl barbituric acid kindly supplied by the Lilly Research Laboratories, Indianapolis, Indiana.

‡ Elemental analysis of the metabolite was performed by the Clark Microanalytical Laboratories, Urbana, Illinois.

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flask containing 250 ml of water. Up to fifty fractions, each containing 5 ml, were collected. The optical densities of the contents of each tube were determined at 262 mμ and 311 mμ* in a Beckman DU spectrophotometer using water as the blank.

It was found that, although no separation of uracil from thiouracil was possible, those fractions in tubes 15 to 30 appeared to contain these two compounds and only relatively small amounts of other substances normally present in urine. The material in all the above fractions exhibited a positive test with Grotes' reagent†, and the ratios of optical densities at 262 mμ/311 mμ in the various tubes ranged from 1·0 to 1·2 or slightly higher. These ratios of optical density indicated the presence of a material other than thiouracil since ratios of the order of 0·6–0·7 were obtained when authentic thiouracil was eluted from Dowex 1-formate resin in a similar manner.

Paper chromatography. Paper chromatography was employed in an attempt to separate the urinary constituents in the eluent from the resin column. Whatman no. 1 filter paper was employed and the solvent system consisted of *n*-butanol saturated with 10 per cent aqueous solution of urea[5]. The combined contents of tubes 15 to 30 were vacuum distilled at room temperature or, in another series of experiments, vacuum desiccated. The concentrate was subjected to ascending chromatography for from 18 to 36 hr, after which time the chromatogram was allowed to dry in air and then examined in the dark under an ultra-violet lamp (Mineralight Ultraviolet Lamp, Model SL 3660). The paper was sprayed with a solution of Grotes' reagent for visualization and identification of any thiouracil present.

The chromatograms, when examined in the dark under an ultra-violet lamp, revealed well-defined spots with an R_f value of 0·30. The R_f values of authentic uracil in this system ranged from 0·28–0·31. After these spots were outlined, spraying the chromatograms with Grotes' reagent revealed another series of spots with R_f values of 0·51–0·56 (average R_f of 0·55). The R_f value for authentic thiouracil in this system is 0·55. The portions of the chromatograms containing the material having an R_f value of 0·30 were clipped out and placed in a stoppered reaction vessel containing 0·01 N NH_4OH. After shaking for 15–30 min a portion of the NH_4OH was removed and analyzed spectrophotometrically. The spectrum of the extract revealed an absorption peak at 262 mμ which is the same as that exhibited by uracil.

In vitro studies. A mince of rat liver, prepared as previously described in the studies on the metabolism of thiamylal, was employed. In some of the experiments a homogenate rather than a mince was utilized. This was prepared as follows: an 11·1 per cent homogenate of liver in 0·15 M KCl was made using a Teflon pestle–pyrex tube tissue grinder. A solution of thiouracil‡ (1 ml), containing approximately 1 mg/ml, was added to an iced beaker containing 9 ml of the minced or homogenized preparation. An aliquot of 1 ml was removed and its content of thiouracil estimated by the method described by Williams *et al.*[6]. The beakers and their contents were incubated in the

* Thiouracil and uracil absorb maximally in the vicinity of 311 mμ and 262 mμ, respectively.

† Grotes' reagent was prepared in the following manner: 10 ml of 5 per cent hydroxylamine HCl and 5 ml of 10 per cent sodium nitroferricyanide were pipetted into a 50 ml Erlenmeyer flask. Ten ml of 10 per cent $NaHCO_3$ was added. Ten minutes later 0·11 ml of bromine was added. When effervescence ceased, 5 ml of 2 per cent phenol was added. The solution was stored in a refrigerator until it turned green. For colour production, the stock reagent was diluted 1 : 20 with 0·2 M phosphate buffer pH 6·8.

‡ Thiouracil, for these studies, was prepared by dissolving the acid form in Krebs–Ringer phosphate solution pH 7·4 to make a 0·1 per cent solution of drug in the buffer.

manner previously described for the studies on thiamylal metabolism. Aliquots again were removed at the end of the incubational period (3 hr) and analyzed for their thiouracil content. The amount of thiouracil which disappeared during the period of incubation was expressed as a percentage of the amount of drug initially present.

With either a mince or homogenate of rat liver, approximately 28 to 35 per cent of the added thiouracil was metabolized in a period of 3 hr. Williams *et al.*[6] employed slices of rat liver and found that approximately 40 per cent of the thiouracil which was added was metabolized in a period of 2 hr.

Ammonia as a metabolic product. During the course of the experiments described herein, Grisolia and Wallach[7] reported that in the biotransformation of dihydrouracil by an enzyme preparation of liver, ammonia was an end product. Experiments were performed to determine whether or not the pyrimidine ring of thiouracil might be enzymically cleaved and ammonia liberated; 2 ml of an 11·1 per cent homogenate of rat liver prepared in isotonic KCl was pipetted into a chilled Warburg vessel containing 0·5 ml of 0·1 N H_2SO_4 in the centre well and 0·5 ml of 0·25 N NaOH in the side arm; 1 ml of a solution of thiouracil, containing 1 mg of thiouracil per ml of Krebs–Ringer phosphate buffer pH 7·4, was added to each flask. Control flasks contained 1 ml of buffer in place of the solution of drug. The flasks were incubated aerobically at 37°C for 4 hr and the reaction was stopped by the addition of the alkali in the side arm to the reaction mixture. Ammonia production was measured by the method described by Battista[8]. When thiouracil was incubated with either a mince or a homogenate of rat liver, an average of 4 μmoles of ammonia was formed in excess of that produced in its absence. The results of these experiments are presented in Table 1. This quantity of ammonia represents approximately 10 per cent of the metabolized drug.

TABLE 1. AMMONIA PRODUCTION BY PREPARATIONS OF RAT LIVER INCUBATED WITH THIOURACIL

Preparation	Ammonia formed μmoles/flask per 4 hr mean (±s.e.)	
	No thiouracil	Thiouracil (1 mg/flask)
Homogenate	3·9 (± 0·7)	7·8 (± 0·2)
Mince	4·4 (± 0·6)	8·2 (± 0·7)

Identification of β-alanine. The formation of β-alanine as a metabolic product of thiouracil was suggested by the significant quantities of ammonia which were formed when the drug was incubated in the manner described previously. Following the incubation period, the contents of the beakers were combined and acidified to a pH of 3–4. The mixture was centrifuged at 4000 rev/min at 0°C for from 15 to 20 min and the supernatant fluid was decanted and concentrated under vacuum at room temperature to a volume of 2 ml. The concentrate was subjected to ascending paper chromatographic analysis. The following solvent systems were employed for the development of the chromatograms: phenol and water in a ratio of 9 : 1; a saturated

solution of phenol in water; *n*-butanol–acetic acid–water (68 : 5 : 27)[9]; lutidine–collidine–water–diethylamine (100 : 100 : 100 : 3)[10]. The chromatograms were developed for 18–20 hr, dried in air and sprayed with an aqueous solution of 1 per cent $CuSO_4$. The chromatograms were again air-dried and sprayed with a 4 per cent solution of diphenylthiocarbazone in reagent grade chloroform. The various R_f values obtained for β-alanine and the metabolite may be seen in Table 2.

TABLE 2. R_f VALUES OF MATERIAL PRESENT IN ACIDIFIED MIXTURES OF HOMOGENATES OR MINCES OF RAT LIVER AFTER INCUBATION WITH THIOURACIL

Solvent system	R_f	
	β-Alanine	Metabolite
Phenol–water (9 : 1)	0·66	0·66
Saturated solution of phenol	0·60	0·62
Colidine–lutidine–water–diethylamine (100:100:100:3)	0·07–0·09	0·07
n-Butanol–acetic acid-water (68 : 5 : 27)	0·18	0·17

Identification of uracil in the incubated mixtures. At the termination of the incubation, the mixtures were combined and alkalinized to a pH of 10 by the dropwise addition of 2·5 N NaOH. After centrifuging the alkaline mixture at 2000 rev/min the supernatant fluid was decanted and concentrated under vacuum at room temperature to a volume of 10–15 ml. A 5-ml aliquot was placed on a resin bed of Dowex 1-formate and eluted as previously described. The combined contents of tubes 15 to 30 were concentrated by evaporation to a volume of 1–2 ml. Aliquots of 0·1–0·2 ml were placed on Whatman no. 1 filter paper and the chromatograms were developed for 18–24 hr in a solvent system consisting of *n*-butanol saturated with a 10 per cent solution of urea in water. Uracil, having an R_f value of 0·30, was visualized with the aid of a Mineralight Ultraviolet Lamp, Model SL 3660. When eluted from the chromatogram with 3–5 ml of 0·01 N NH_4OH and analyzed spectrophotometrically, an ultra-violet absorption maximum in the vicinity of 262 mμ was revealed. Maximum and minimum absorption peaks were the same as authentic uracil.

Thiouracil-2-C^{14}. Previous studies showed that uracil could be identified as one of the products formed following incubation of thiouracil with either a homogenate or a mince of rat liver. Uracil also was identified in the urine of animals receiving thiouracil. Further studies were undertaken to determine if the uracil found was derived from the desulphuration of the thiouracil or merely resulted from the replacement of uracil by thiouracil in the cell, e.g. nucleotides, or the inhibition of uracil utilization by the cell. Employing thiouracil-2-C^{14} as an aid in the identification and quantitative estimation of uracil, the extent to which desulphuration participates in the biotransformation was investigated.

The synthesis of thiouracil-2-C^{14} was similar to that described by Wheeler and Liddle[11]. The sodium salt of ethyl formyl acetate was prepared according to the

directions of Wislicenus[12]. A total of 7·083 g of thiouracil was obtained. This represented a 40·1 per cent yield on a theoretical molar return of 17·62 g. To determine the yield with respect to activity, the synthetic material was compared with the original standard of thiourea-C^{14}*. The total activity of the synthetic product was calculated to be $1{\cdot}24 \times 10^7$ counts/min. This was equal to a yield of 35·5 per cent on the basis of incorporation of radioactivity. The material melted with decomposition at 336°–340°C. A sample was dissolved in borate buffer at pH 9·4, and the ultra-violet absorption spectrum was obtained using buffer as the blank. Maximum and minimum points agreed with authentic thiouracil as did the ratio of the optical density at 311 mμ to that at 262 mμ. This ratio for the synthetic product was 0·661 and for authentic thiouracil (Nutritional Biochemicals Corporation, Cleveland, Ohio) it was 0·667.

Urinary excretion of radioactivity. Preliminary experiments were performed to obtain a quantitative estimate of the percentage of radioactivity excreted in the urine in a 24-hr period by the rat following administration of a single dose of thiouracil-2-C^{14}. Twenty-four rats, weighing between 170–180 g, were injected intraperitoneally with the preparation containing thiouracil-2-C^{14}† in a dose of 20 mg per animal. Two animals were placed in each metabolism cage and allowed free access to food and water. Urine was collected for a 24-hr period, after which each cage was washed with water. The washings were either added to the collected urine or examined separately. Radioactivity measurements of the material in the urine of rats receiving thiouracil-2-C^{14} revealed that in 24 animals an average of 79·2 per cent (s.e. $= \pm 2{\cdot}2$ per cent) of the injected radioactivity was excreted during a 24-hr period. These results represent minimal values for excretion of radioactivity since no correction was made for self-absorption error due to the plating of urinary solids, e.g. weight correction.

In vivo metabolism of thiouracil-2-C^{14}. Six male rats of the Holtzman strain, weighing between 180–200 g were hydrated with water (20 ml/kg) 20–30 min prior to an intraperitoneal injection of 20 mg of thiouracil-2-C^{14}. The animals were paired, placed in three metabolism cages and allowed free access to food and water. Urine was collected for 48 hr and each cage was washed with 50 ml of water. After washing with chloroform and ethylene dichloride, as described previously, the urine was concentrated to a volume of approximately 20 ml by vacuum desiccation. Five 0·1 ml aliquots of the chloroform and ethylene dichloride extracts were examined for their radioactivity. A 5 ml aliquot of the concentrated aqueous phase which had been extracted with organic solvents was subjected to ion exchange chromatography as described previously. Following the collection of fifty fractions of 5 ml each, 15 ml of concentrated formic acid were passed over the resin bed. A 1 ml aliquot from each fraction was tested with Grotes' reagent. An aliquot of 0·1 ml was removed from each tube, and its radioactivity measured. The material in those tubes (18 to 37) which exhibited a positive test with Grotes' reagent was combined and evaporated to dryness in a vacuum desiccator. The residue was dissolved in 1·5 ml of 0·01 N NH_4OH and 0·1 ml aliquots were spotted on $1\frac{1}{2}$ in. wide strips of Whatman no. 1 filter paper. Ascending chromatography was performed for a period of 22 hr in a system of *n*-butanol saturated with a 10 per cent aqueous solution of urea. The paper chromatograms were dried in air and analysed for radioactivity in the following manner: the strips were placed in a strip

* Thiourea-C^{14} was obtained from Tracerlab, Inc., Boston, Mass. The specific activity of the material was 1·00 mc/m-mole.

† For these studies, the synthesized material was diluted 1 : 9 with non-labelled thiouracil. Solutions were prepared as described previously.

feeder and moved 1 cm at a time under an end-window Geiger–Mueller tube. The counting time was 10 min at each centimetre interval. Following the measurement of radioactivity on each paper chromatogram, the paper strip was sprayed with Grotes' reagent.

Radioactivity measurements on the chloroform and ethylene dichloride extracts of the urine revealed that an average of 1·91 per cent of the total radioactivity was removed by chloroform and 6·2 per cent was extracted into ethylene dichloride. The results of fractionation studies on the concentrated urine, after extraction with the organic solvents, are presented in Fig. 3. Three distinct peaks of radioactivity are

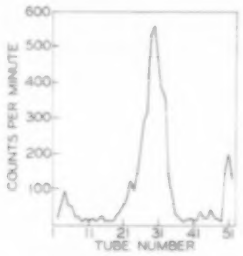

FIG. 3. Fractionation on Dowex 1-formate resin of C^{14}-labelled material in concentrated urine after organic solvent extraction.

present. The first peak is between tubes 1 and 9. The greatest amount of activity is in the peak represented by tubes 18 to 37. A third peak represents radioactive material which was eluted from the resin column with concentrated formic acid. Aliquots from each tube in the fractionation tested with Grotes' reagent revealed that only material in tubes 20 to 36 exhibited a positive test. Since thiourea when present will normally appear in tubes 4 to 9 and will cause a blue coloration of Grotes' reagent, it appears that the material which is present in tubes 1 to 9 is not thiourea. This again suggests that a direct splitting of the pyrimidine ring of thiouracil to yield thiourea and an acrylic acid derivative does not occur to any appreciable extent.

Radioactivity measurements of the developed paper strips revealed that only two areas of radioactivity were present. One of these, possessing an R_f value of 0·33, accounts for approximately 16·5 per cent of the radioactivity. The other, having an R_f value of 0·52, contains the remaining 82·6 per cent of the radioactivity. Results of such an experiment are illustrated in Fig. 4 and represent the average values obtained from three paper chromatograms. When sprayed with Grotes' reagent, a blue coloration appeared in the area of the paper containing material possessing an R_f value of 0·52. The spots containing the material with an R_f value of 0·33 were clipped out, placed in a reaction vessel and extracted with a 0·01 N solution of NH_4OH. Aliquots of the alkaline solution were analysed in a Beckman DU spectrophotometer. An ultraviolet absorption spectrum with a maximum at 262 mμ was obtained. This is the wavelength at which authentic uracil absorbs maximally under similar experimental conditions.

Thiopental metabolism

Since previous experiments had demonstrated that certain cruder preparations of rat liver (a mince or a fortified homogenate) were capable of converting various thio-

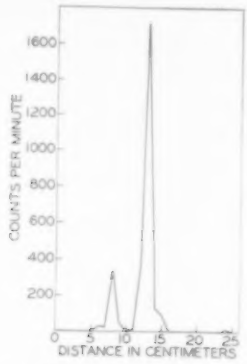

FIG. 4. Separation on Whatman no. 1 paper of radioactive material in tubes 18 to 37. System: *n*-butanol saturated with a 10 per cent aqueous solution of urea. Development time: 22 hr.

pyrimidines (thiopental, thiamylal, thiouracil) to their corresponding oxygen analogues experiments were performed to determine the intracellular distribution and some of the characteristics of the enzymic system responsible for such a biotransformation. Livers were obtained from rats as previously described and placed in chilled isotonic KCl solution. The tissue was blotted between layers of Whatman no. 4 filter paper and, after weighing, 5 ml of fresh isotonic KCl solution was added for each 1 g of liver. The tissue was homogenized within a period of 1 min in an iced Teflon pestle–pyrex tube tissue grinder. The homogenate was centrifuged at $9000 \times g$ at 0–3°C for 10 min. The supernatant fluid was decanted into an iced, graduated cylinder. Sufficient isotonic KCl was added to the precipitated matter in the tube to make a 25 per cent mixture, and this was resuspended by re-homogenization. The resuspended material was centrifuged again at $9000 \times g$ and the resulting supernatant fluid was combined with the first supernatant fraction and utilized for the studies to be described. Thiopental* was added last to the incubation mixture (see Table 4) and a 1 ml aliquot was removed prior to incubation and analysed for its concentration of thiobarbiturate by the method described by Brodie *et al.*[3]. The beakers were incubated for a period of 3 hr as previously described. Aliquots were again removed after the incubational period. The disappearance of drug in these experiments was expressed as μmoles of thiopental metabolized per 3 hr per g of liver tissue.

In an attempt to establish further the importance of the added cofactors, experiments were performed in which various components of the system were omitted. To ascertain the effect of incubation in an anerobic atmosphere parallel experiments were performed with a divided hood in a Dubnoff metabolic shaking incubator utilizing equal flow rates (5 ft^3/hr) of oxygen and nitrogen. In the experiments designed to determine the effect of cyanide ion on the system, NaCN was prepared in phosphate buffer pH 8·0. One ml of the solution was added to 25 ml Erlenmeyer flasks containing the system. The flasks were stoppered immediately following the removal of aliquots and incubated. Control flasks contained 1 ml of phosphate buffer, pH 8. It was found that stoppering the flasks did not markedly affect the rate of disappearance of drug. Since other investigators[13–16] have reported that β-diethylaminoethyl

* Thiopental solution was prepared in a manner similar to that used in the preparation of thiamylal, namely, the acid was solubilized by the dropwise addition of 2·5 N NaOH. The solution was made to volume with water.

diphenylpropylacetate hydrochloride (SKF 525-A) inhibits the hepatic metabolism of a number of drugs with widely different structures, it was of interest to determine whether or not this drug also would inhibit the metabolism of thiopental. In these studies SKF 525-A was dissolved in isotonic KCl and employed at a final concentration of 2×10^{-4} M. A study of the rate of metabolism of thiopental by hepatic homogenates of various species also was undertaken. For this work, combined microsomal and soluble fractions from the livers of the rat, mouse, dog, rabbit, guinea pig and pigeon were used.

TABLE 3. METABOLISM OF THIOPENTAL BY MICROSOMES PLUS THE SOLUBLE FRACTION FROM THE LIVER OF THE RAT

System	Thiopental metabolized (μmoles)
Complete*	0·25
Minus TPN	0·08
Minus glucose-6-phosphate	0·01
Minus magnesium chloride	0·02
Minus nicotinamide	0·00
Minus supernatant	0·00
Plus cyanide 10^{-2} M	0·20
Incubated anaerobically	0·01
Plus SKF 525A 2×10^{-4} M	0·01

* The complete system contained microsomes plus supernatant (5·0 ml), 0·5 μmole thiopental (1 ml), 0·1 M phosphate buffer pH 7·4 (1·0 ml), 0·5 μmole TPN (0·5 ml), 5·0 μmoles glucose-6-phosphate (0·5 ml), 50 μmole magnesium chloride plus 100 μmoles nicotinamide (2·0 ml). Incubation time was 3 hr at 37–38 °C in an atmosphere of oxygen.

In Table 3 are illustrated the results of a typical experiment; under these conditions nicotinamide, magnesium ion, supernatant (microsomes plus soluble fraction) and aerobic conditions are required for a maximal rate of metabolism of thiopental. The omission of triphosphopyridine nucleotide (TPN) caused a reduction in the metabolic rate of approximately 75 per cent. SKF 525-A, at a concentration of 2×10^{-4} M, inhibited the system markedly, while cyanide ion, at 10^{-2} M, decreased the metabolic conversion of thiopental by only 20 per cent.

The rate of metabolism of thiopental by the active fractions (microsomes plus soluble fraction) of the livers of various species are illustrated in Table 4. It can

TABLE 4. METABOLISM OF THIOPENTAL BY THE ACTIVE FRACTIONS (MICROSOMES PLUS SOLUBLE FRACTIONS) OF THE LIVER OF VARIOUS SPECIES

Animal	Number of animals	Thiopental metabolized (μmoles/g per 3 hr)*
Rat	12	0·25
Mouse	8	0·17
Dog	2	0·16
Rabbit	2	0·18
Guinea pig	3	0·09
Pigeon	4	0·05

* Active fractions obtained from 1 g of liver.

be seen that the rate of metabolism is greatest in the case of the rat. Such a preparation from the livers of the rabbit, mouse and the dog were about equally active, possessing about 70 per cent of the activity of rat liver. Preparations of hepatic tissue from the guinea pig and pigeon were found to be approximately one-third and one-fifth, respectively, as active as those of the rat.

Studies with microsomes from rat liver. A microsomal plus soluble fraction was prepared as previously described. (In some of the experiments the liver homogenate was prepared in a solution of isotonic sucrose rather than KCl.) The preparation was acidified with 0·1 N acetic acid to pH 5·4 and centrifuged for 10 min at 25000×*g* at 0°C. The supernatant fluid was decanted and the residue, containing the microsomes, adjusted to pH 7·4 with a 5 per cent solution of Na_2CO_3. Sufficient iced isotonic KCl solution was added to yield a preparation containing in 1 ml the microsomes obtained from 1 g of liver. Aliquots of 1, 1·5, 2, 2·5 and 3 ml were pipetted into chilled beakers. To these beakers an equal volume of the supernatant was added. Thus, in beaker no. 1 there was 1 ml of the microsomal preparation plus 1 ml of supernatant material (soluble fraction from 1 g of liver); beaker no. 2 contained 1·5 ml of microsomes plus 1·5 ml of supernatant material, etc. In addition, each beaker contained 0·5 μmole TPN, 5 μmoles glucose-6-phosphate, and 2 ml of the nicotinamide–$MgCl_2$ solution. Sufficient isotonic KCl was added to each beaker to bring the volume of reaction mixture to 10 ml. The incubation was carried out for 3 hr in the usual manner. Results of experiments in which 1 μmole of thiopental was incubated with increasing amounts of a microsomal preparation from the liver of the rat are shown in Fig. 5. These experiments demonstrate that a linear relationship exists between the amount of thiopental metabolized and concentration of active liver fraction when less than 6 ml of enzyme preparation was employed.

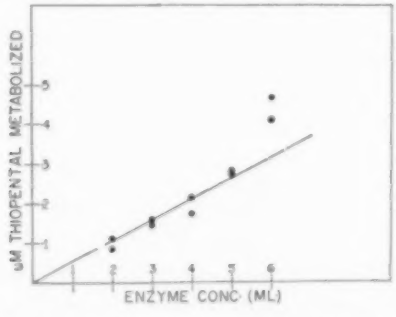

FIG. 5. Relationship between concentration of enzyme preparation of rat liver microsomal fraction and amount of thiopental metabolized. Enzyme concentration is expressed in ml units and refers to the combined volumes of microsome and soluble fractions.

Others workers[13–17] have indicated that the presence of reduced triphosphopyridine nucleotide (TPNH), rather than triphosphopyridine nucleotide (TPN), is required for the metabolism of certain drugs by systems similar to the one employed in these experiments. That such a situation might exist in the metabolic desulphuration of thiopyrimidines suggested the following experiment. Thiopental was incubated with microsomes and TPNH which was prepared by the procedure of Kaplan *et al.*[18]. In

these studies, experiments were also performed in which each of the cofactors of the system was omitted, one at a time. In addition to this, the effects of anaerobic, conditions and SKF 525-A (2×10^{-4} M) were determined. Shown in Table 5 are results

TABLE 5. METABOLISM OF THIOPENTAL BY MICROSOMES FROM THE LIVER OF THE RAT

System	Thiopental metabolized (μmoles)
Complete*	0·31
Minus TPNH	0·03
Minus magnesium chloride	0·01
Minus nicotinamide	0·29
Incubated anaerobically	0·02
Plus SKF 525A 2×10^{-4} M	0·01

* The complete system contained microsomes obtained from 1 g liver (5·0 ml), 0·5 μmole TPNH (0·5 ml), 50 μmoles magnesium chloride (0·5 ml), and 0·1 M phosphate buffer, pH 7·4 (3·0 ml). Incubation time was 3 hr at 37–38 °C in an atmosphere of oxygen.

obtained from experiments in which thiopental was incubated with microsomal fractions of rat liver. In these experiments SKF 525-A, anaerobic conditions and deletion of magnesium ion or TPNH caused a marked reduction in the amount of thiopental metabolized.

Identification of pentobarbital as a product of the metabolism of thiopental by hepatic microsomes. Rat liver (56 g) was obtained from 9 female rats and the microsomal fraction was prepared in the manner previously described. This was added to 125 ml of isotonic KCl solution in a 2000 ml beaker. The final reaction mixture contained 40 ml of TPNH (2 μmoles/ml), 80 ml of a solution containing nicotinamide (50 μmoles/ml) and $MgCl_2$ (25 μmoles/ml), 40 ml of a solution of thiopental (4 μmoles/ml) and 75 ml of 0·1 M phosphate buffer, pH 7·4. The mixture was thoroughly stirred and then was incubated at room temperature (about 27 °C) for 3 hr in air. Following the incubation, the mixture was handled in a manner similar to that described by Winters *et al.*[1] for the isolation of pentobarbital when thiopental was incubated with a mince of rat liver. From this experiment there was obtained 1·2 mg (11·3 per cent of the total drug metabolized) of crystalline material which was subjected to ultra-violet and infra-red analysis. The spectra of authentic pentobarbital and this metabolic product revealed no significant differences.

DISCUSSION

The work described indicates that there exists in the livers of various animals a biochemical mechanism which is capable of converting various thiopyrimidines to their corresponding oxygen analogues. Shideman *et al.*[19] and Dorfman and Goldbaum[20] have suggested that the liver is the main site of the catabolism of certain barbiturates and thiobarbiturates. Other workers[21, 22], although postulating desulphuration as a possible route in the metabolic transformation of thiobarbiturates, were unable to demonstrate the formation of the oxygen analogues. This was partly due to lack of adequate techniques and the fact that the metabolic products, as later demonstrated,[23]

may be oxidized at rates comparable to those of the parent compounds. The work of Raventos[24] and of Winters *et al.*[1] indicates that thiobarbiturates can be metabolized by a reaction involving desulphuration. During the metabolism *in vitro* of S^{35}-labelled thiopental by a mince of rat liver, the major portion of the labelled sulphur was identified as inorganic sulphate.[1] Others [21] have found that thiopental-S^{35}, when administered to animals, is metabolized to yield approximately 80 per cent of the radioactivity in the urine in the form of inorganic sulphates. Employing thiamylal, thiouracil and thiopental it was found that the major metabolites formed *in vitro* are the oxygen analogues, secobarbital, uracil and pentobarbital, respectively. In the case of thiamylal and thiouracil, this is believed to be the first time that the oxygen analogues have been demonstrated as metabolites.

The metabolic formation of β-alanine and ammonia when thiouracil is incubated with preparations of liver *in vitro* supports the concepts of pyrimidine metabolism which have been proposed by Rutman *et al.*[25], Fink *et al.*[26] and Grisolia *et al.*[27]. Since no thiourea could be demonstrated in the experiments with thiouracil *in vitro* or *in vivo*, it would appear that a primary cleavage of the thiopyrimidine ring with the formation of thiourea and an acrylic acid derivative does not occur. Therefore, it is suggested that the route of metabolism of thiouracil follows the series of reactions indicated in Fig. 6.

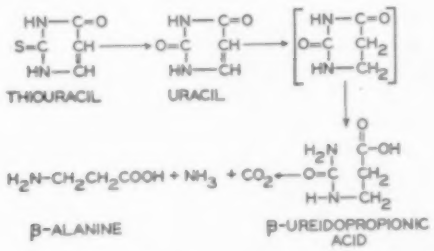

FIG. 6. Proposed route for the metabolism of thiouracil by the rat.

The enzymic mechanism which converts thiopental to pentobarbital and probably thiamylal and thiouracil to their oxygen analogues appears to be localized in the microsomal fractions of liver and possesses several unusual features. Thiopental and certain other thiobarbiturates have been shown to be metabolized by a crude, cell-free, fortified homogenate of rat liver by Gould and Shideman[28]. They clearly demonstrated that of the tissues which they studied the greatest activity resided in the liver. No attempt was made to fractionate or purify the system nor were studies performed to determine the fate of thiopental. Results of these authors' studies indicate that the conversion of thiopyrimidines to their oxygen analogues is accomplished by an enzyme or enzyme system which is unusual in requiring TPNH and oxygen. Since in these studies the soluble fraction and TPN will replace TPNH, it appears that the soluble fraction is required to maintain the TPN in a reduced state, either through the glucose-6-phosphate dehydrogenase system or some other system generating this cofactor. These findings are in agreement with those of other workers[13, 17] who have found that TPN in the absence of the soluble fraction is effective only in the reduced form. The finding that cyanide does not block thiopental metabolism indicates that

the electrons from TPNH are not routed to oxygen along the cytochrome–*c*–cytochrome oxidase chain, as it is presently understood. It is possible that a cyanide-insensitive cytochrome enzyme may still be involved in this transfer.

It may be inferred from the results obtained that a major pathway in the metabolism of thiopental, thiamylal and thiouracil is desulphuration. This reaction is involved initially and accounts for a major portion of metabolized drug. Since the oxygen analogues of the thiobarbiturates have never been isolated from the tissues or fluids of man, it is believed that further work is necessary to identify the other metabolic products which result upon further catabolism of the oxygen analogues. Recently, Winters[29], studying the metabolic fate of thiopental in the rat, dog and man, has proposed that the major transformation product in man is the carboxylic acid derivative and in dogs and rats the major metabolites are the alcoholic derivatives of pentobarbital. This hypothesis is based on a comparison of the R_f values of unidentified materials extracted from the urine of animals receiving thiopental with values reported by other workers for previously isolated metabolites. However, it is believed that further work is necessary to isolate and characterize these transformation products.

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FAILURE OF THE URETHANE ANTAGONIST—LYSERGIC ACID–DIETHYLAMIDE (LSD–25)—TO INHIBIT LUNG CARCINOGENESIS OR THE INITIATING PHASE OF SKIN CARCINOGENESIS IN MICE

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Abstract—The fact that LSD-25 (lysergic acid-diethylamide) has been shown to be a powerful antagonist against the anaesthetic and other neurological effects of urethane, raised the question of whether it would also antagonize its carcinogenic action. The present investigation failed to elicit any antagonism by LSD-25 against the carcinogenic action on the lung, or against the initiating phase of skin carcinogenesis, induced by urethane in mice. The claim that the antagonism between LSD-25 and urethane is "complete" is therefore not substantiated. The results indicate, on the contrary, that the mechanism involved in urethane carcinogenesis is different from that involved in its neurological effects.

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In a pharmacological study of the action of LSD-25 (lysergic acid-diethylamide) on sympathetic ganglia in cats under urethane anaesthesia, Broghammer, Takagi and Schaefer[1] observed a striking antagonism between LSD-25 and urethane action, not only on the peripheral ganglia but also on the central nervous system. The antagonism operated when the LSD-25 was administered shortly before the urethane, but not when given simultaneously with or shortly after it. The antagonistic effect was demonstrable even under conditions when the dose of LSD-25 was 1/7500th of the dose of urethane, but was nevertheless described by them as "competitive".

Urethane is also a potent anaesthetic for the mouse, and in view of its known carcinogenic action on the lungs[2], and its more limited "initiating" action in relation to skin carcinogenesis, in the mouse (i.e. requiring subsequent "promoting" action for complete carcinogenesis),[3, 4] it was thought desirable to test whether LSD-25 also antagonized these carcinogen-related effects of urethane, even though complete antagonism in the "competitive" sense seemed unlikely on *a priori* grounds (in view of the striking difference in chemical structure between the two substances, and the great divergence in dosage required for the antagonism described by the previous authors[1]).

METHODS AND MATERIALS

The animals used in these experiments were male and female mice of the Swiss strain, bred in these laboratories by brother–sister mating for from twenty-two to twenty-four generations, and were 6 weeks old at the start of the experiment. They were kept in transparent plastic cages, and housed in an air-conditioned room at 21–23°C, and fed on *Purina Laboratory Chow* and water *ad libitum*.

In preliminary tests, the per cent mortality v. dose of intravenously injected LSD-25 was determined for the strain of mice used, to get an approximation of the maximum safe dose. The values obtained (55 mg/kg for $L.D._{50}$ and 30–40 mg/kg for $L.D._{0}$) proved to be very close to those obtained by Cerletti[5]. The dose subsequently chosen for the actual experiments was, therefore, 30 mg/kg, or 0·6 mg per 20 g mouse.

A 1 per cent solution of LSD-25 ("Delysid", supplied by Sandoz S.A., Basle, as "pure powder"), in double distilled water, was freshly prepared immediately prior to its use, and 0·06 ml of it injected per animal by the tail vein. (In view of its light sensitivity, the solution was made up and kept in subdued light.) Three hours after its injection, 20 mg of urethane, as a 10 per cent solution in distilled water, was injected intraperitoneally in each animal. The control mice received the same dose of urethane without prior LSD-25 treatment.

TABLE 1. EFFECT OF LSD-25 ON URETHANE ACTION IN MICE, WITH RESPECT TO (a) INITIATING ACTION ON SKIN, AND (b) CARCINOGENIC ACTION ON THE LUNGS

Sex	Initiating action*	Promoting action†	Skin tumours			Lung tumours	
			Mice bearing papillomas/ survivors	Average no. papillomas per mouse	Average latent period‡ (weeks)	Mice bearing adenomas/ survivors	Average no. adenomas per mouse
F	U+LSD-25	Croton oil	20/21 95%	3·3 ± 0·4	9	20/20	4·3 ± 0·4
M			18/22 81%	1·9 ± 0·3	11	18/18	2·8 ± 0·4
F and M			38/43 88%	2·6 ± 0·3	10	38/38	3·6 ± 0·3
F	U control	Croton oil	23/25 95%	1·9 ± 0·3	11	23/23	3·6 ± 0·3
M			9/12 75%	1·9 ± 0·4	10	12/12	3·8 ± 0·5
F and M			32/37 86%	1·9 ± 0·4	11	35/35	3·7 ± 0·3

* U = 20 mg urethane intraperitoneally, single injection.
LSD-25 = 0·6 mg lysergic acid–diethylamide intravenously, single injection 3 hr prior to urethane injection.

† Croton oil (5%) in liquid paraffin, applied twice weekly to skin for 20 weeks, starting 14 days after initiating action.

‡ Figures refer to *first* tumours per animal.

After an interval of 14 days, all the animals began to receive standard promoting action, consisting of applications of a 5 per cent suspension of croton oil in medicinal liquid paraffin, to an area of skin about 2×2 cm, in the dorsal region of the back. These applications were made with a glass rod, after clipping the hair with scissors, and repeated twice-weekly for 20 weeks.

The resulting skin tumours were charted at their first appearance, and fortnightly thereafter. Papillomas that regressed within 2 weeks of their first appearance were not listed in the final records. At the end of the experiment, the animals were killed, and careful note taken at autopsy of the number of lung adenomas in each animal.

RESULTS

From preliminary tests with LSD-25 alone, an indication of the effectiveness of the dose used in the actual experiment (0·6 mg per 20 g mouse) was evidenced by the symptoms of intense alertness, high excitability, increased motor activity, acceleration of the heart rate, increased rate and depth of respiration, transitory generalized tremors, pupillary dilation and pilo-erection. Many of these effects persisted for several hours, with a peak at about 2 hr after injection. When this dose of LSD-25 was given 3 hr before injection of 20 mg of urethane (i.e. in the experiment proper), the anaesthetic effect of the latter was entirely antagonized.

The results of the experiment proper, with respect to carcinogenesis, are summarized in Table 1, from which it will be seen that the average latent period of skin tumours, and the total number of skin tumours and of lung adenomas, were very similar in the experimental and control groups, the minor differences lacking statistical significance. (If anything, the tumour yield in the skin was higher in the experimental group.) In short, LSD-25 failed to antagonize, or inhibit in any way, the carcinogenic action of urethane on the lungs or its initiating action on the skin.

DISCUSSION

Under the experimental conditions used here, LSD-25—a pharmacological antagonist against urethane action with respect to its anaesthetic and other neurological effects—failed to inhibit its carcinogenic action on the lungs or its initiating action in connexion with skin carcinogenesis in mice. In considering the significance of these negative findings, the question of dosage is naturally critical. It is pertinent to point out, in this connexion: (a) that the amount of LSD-25 used (0·6 mg per 20 g mouse) represented the "maximum safe dose" and more than half the $L.D._{50}$ for the mice used; (b) that the pharmacological effects elicited by this dose were very pronounced; and (c) that when this amount of LSD-25 was administered in conjunction with urethane, the anaesthetic effect of the latter was completely prevented. Consequently, any antagonism which LSD-25 might have against the carcinogenic action of urethane should have been readily demonstrable in the present experiments. The fact that no inhibition was observed shows that LSD-25 completely lacks any antagonism against the carcinogenic action of urethane.

Two conclusions may be drawn from these results: (a) that the mechanism of carcinogenic action of urethane on the lung, and of its initiating action on the skin, is different from that involved in its general pharmacological effects, and (b) that the claim of Broghammer *et al.*[1], that the antagonism between LSD-25 and urethane is complete, in the "competitive" sense, is unjustified. The latter conclusion is hardly surprising, in view of the striking difference in chemical structure between the two compounds (cf. Roblin[6]; Work and Work[7]; Woolley[8]; etc.), and also in view of the great divergence in dosage between LSD-25 and urethane required for the antagonism of the neurological effects described by the previous investigators[1].

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HISTAMINE-LIBERATOR 48/80 AND BASOPHILIC LEUKOCYTES*

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Abstract—When introduced *in vivo*, histamine-liberator 48/80 produces an abrupt disappearance of basophil leukocytes in the peripheral blood of rabbits and chickens. This disappearance is not due to migration into the tissues, but to degranulation in the blood stream. This process is generally coincident with the development of the shock-like state. A similar phenomenon is seen with the introduction of snake venom *in vivo* and *in vitro*. Colchicine produces a basophilic leukocytosis upon repeated subcutaneous administration.

THE presence of histamine in blood cells and fixed tissue cells, and the participation of histamine-release in anaphylaxis and related phenomena have been well established concepts for some time. After Alam *et al.*[1] discovered that curare would release histamine from tissues, a variety of related and unrelated compounds were discovered to have this same property.[2, 3] The general topic of histamine-release by simple chemicals has been reviewed recently by Paton[4]. Among the now large and varied list of compounds that possess this property, there are a few which are extremely specific, and with which histamine-release accounts for the bulk of the pharmacological properties. Among these specific histamine-liberating drugs, the substituted phenethylamine low polymer 48/80 has had the widest usage in the study of problems of histamine release. The pharmacology of this drug has been described by Dews *et al.*[5]

Numerous investigators have studied the effect of compound 48/80 on the release of granules by tissue mast cells,[6–8] and it appears that these cells may extrude their granules without cell disruption or death. Recently, Asboe-Hansen and Glick[9] failed to show this granule discharge *in vitro*, and claim that 48/80 produces only a destaining phenomenon, without cell disintegration or degranulation.

Since the basophilic granulocyte of circulating blood has a considerable cytochemical relationship to the mast cell of connective tissues, it is of interest to study the effect of histamine-liberators upon this cell. Basophilic leukocytes contain histamine,[10] and there is evidence that 48/80 causes a fall in the number of basophils in circulating blood.[11, 12] The object of this study is to determine the acute and chronic effects of 48/80 upon the numbers of basophils in the blood of rabbits and chickens, then to record photographically the cytological effects of 48/80 upon these cells.

MATERIALS AND METHODS

Adult male albino rabbits, weight from 2·5 to 3 kg, and hybrid cockerels, weight

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from 1 to 1½ kg were used; 48/80*, dissolved in physiological saline solution, was given intravenously in the ear and wing veins, respectively. Blood samples for counting or for supra-vital examination were collected from ear or wing veins on the side opposite to that injected. Blood sampling from the same portion of a vein at frequent intervals was avoided. Counting of basophils was done by a modification of the method of Moore and James[13]. The modification consists in the use of a diluting fluid consisting of: 40 ml of 0·1 M phosphate buffer, pH 7·6, 11 ml ethanol, and 1·4 ml of 1 per cent aqueous toluidine blue. Saponin was not found necessary, and omitting it led to a marked decrease of precipitate in the counting chamber.

Supra-vital preparations of rabbit blood for cytological observation of the living cells were made in the conventional manner on slides coated with a film of Janus green and neutral red, whose thickness was adjusted so that the rabbit leukocytes took up the dye rapidly, but without any interference with their vital activities. Basophilic leukocytes were examined under bright light and phase contrast, and numerous micro-cinematographs were taken under oil immersion, 970 × phase contrast, 8 frames/sec with Ektachrome colour film. Still photographs in this report were made from these movie frames. A small group of rabbits were treated with colchicine; the latter was dissolved in physiological saline, and injected subcutaneously, 1 mg/kg. Saline-injected control rabbits and cockerels also were used.

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RESULTS

Figure 1 shows the time course of the decrease in number of basophils in the cockerel. It can be seen that this process begins quite rapidly. In a number of chickens, a blood sample taken 1 min after injection of 2 mg/kg of 48/80 showed a complete absence of

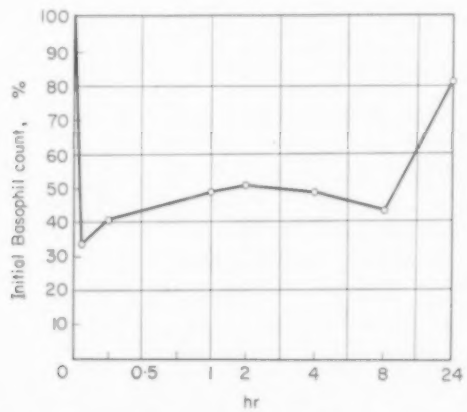

FIG. 1. Acute effects of 48/80 in cockerels (dose, 2 mg/kg i.v.). Each point represents the average of ten animals.

basophils in the counting chamber. We attribute this to the prevention of metachromatic staining, as observed by Asboe-Hansen and Glick[9] *in vitro*. An actual disappearance of cells seems to begin in about 5 min, at the same time the shock-like reaction to 48/80 began. The approximate 50 per cent depression of basophil count

* Generously provided by The Burroughs-Wellcome Co.

lasted for at least 8 hr, and complete recovery had not occurred after 24 hr. Similar results were seen with the injection of 1 mg/kg into rabbits. A group of three rabbits were then treated twice weekly with this dose of 48/80, to determine the effect of repeated administration upon the acute depletion of basophils. It can be seen (Fig. 2) that over a 5-week period there was essentially no change in the abrupt disappearance of these cells that occurs 5–10 min after the administration of the drug. The apparent increase of basophils in the trial at the end of the second week represents an unexplained marked basophilia on the part of one rabbit of the group. This was the only increase seen in ten trials with each of three rabbits, thirty trials in all (Fig. 2).

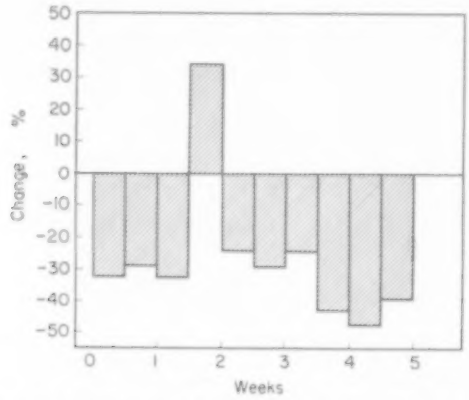

FIG. 2. Acute effects of 48/80 on the basophil count of male rabbits. Dose, 1 mg/kg given i.v. twice weekly to three rabbits. Counts were made just before and 5–10 min after the injections. Saline controls showed no change.

We were interested in comparing the chronic response of basophils to 48/80 with the response to colchicine, a drug that has been shown to have marked destructive effects on mast cells,[14, 15] as well as effects on basophilic leukocytes.[16] Figure 3 shows that 48/80 and colchicine produced markedly different basophil responses upon continued administration for a period of weeks. With the former there was no important change either in absolute or relative basophil counts, while with colchicine, beginning at the third to fourth week, there occurred both an absolute and a relative basophilia, so that as much as a 20 per cent or even higher basophil count was observed in contrast to the 3–6 per cent which our animals normally showed.

Finally, in attempting to observe the fate of basophils as they disappear rapidly after the administration of 48/80, we made supra-vital preparations of rabbit blood within the first 5 min after administering doses of 48/80 of 1–1·5 mg/kg. These animals began to develop the shock-like response to the drug within 10 min after administration, and at the same time we could observe massive destruction or degranulation of basophils which we recorded in colour movies*. Figure 4 is composed of a set of stills selected to show the sequence of changes in a typically reacting cell. Watching the living cells, we observed first an increase in the cytoplasmic streaming activity, which suddenly stopped immediately preceding the most dramatic changes, i.e. either the development of large

* Movies referred to were shown at the meeting of the Ohio Valley Section, Society for Experimental Biology and Medicine, November 1958.

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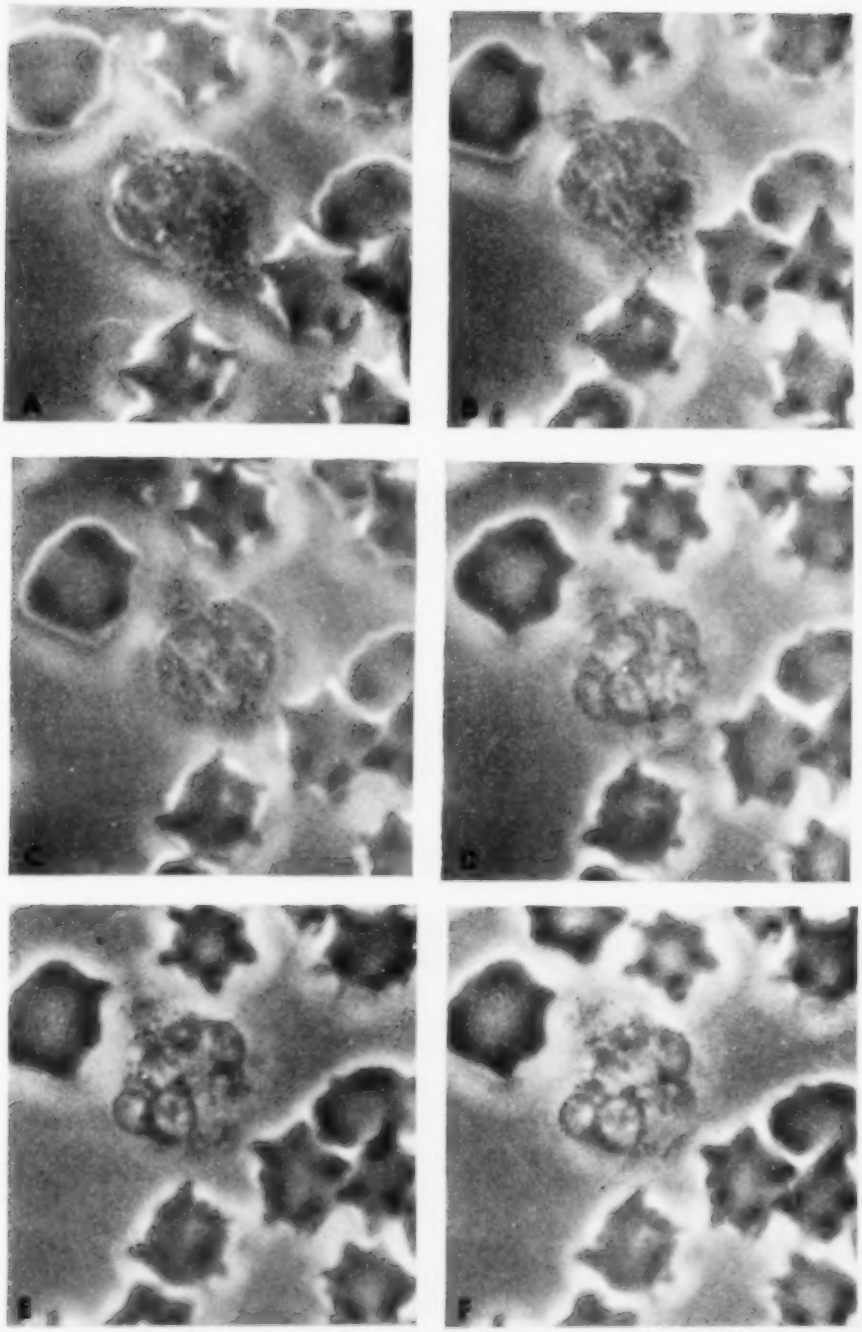

FIG. 4. Degranulation of a rabbit basophil leukocyte produced by 48/80. Dose 1 mg/kg i.v. Supravital preparation of rabbit blood made 5 min after injection. Series of photographs at 30 sec intervals from a movie film taken at 970× with phase contrast; (A) normal granulated basophil; (B) (C) granules show indistinctness of outline; (D) (E) degranulation proceeding rapidly, with clear spaces left to indicate the site of the granules; (F) degranulation almost complete.

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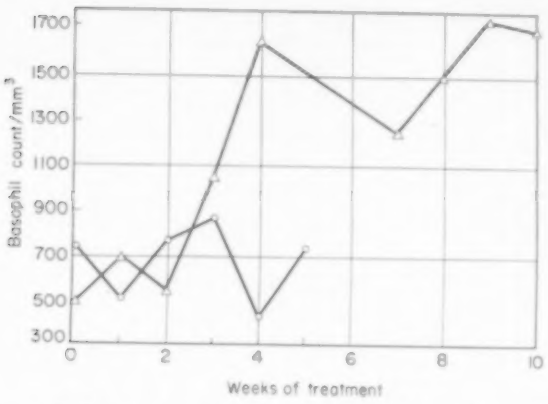

FIG. 3. Effects of repeated administration of 48/80 and colchicine upon basophil leukocytes of male rabbits. Injections were made twice weekly in three rabbits. Counts were taken just before injections.
△—Colchicine — 1·0 mg/kg subcut
○—48/80 — 1·0 mg/kg I.V.

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cytoplasmic vacuoles, or rapid disappearance of the specific basophilic granules, or both. Once begun, the disappearance of granules took place over a period of about 90 sec, and at the conclusion the cells were left with an essentially clear cytoplasm. It appeared that the cell membrane remained intact, although these cells have not been observed to resume any amoeboid motion or cytoplasmic streaming once the degranulation has been completed. Occasionally it appeared that intact granules were ejected from the cell, but this was exceptional, the usual picture being one of intracellular rupture of granules along with swelling of the cell or vacuole formation. It is important to emphasize that in our numerous control supravital preparations this sequence of changes was not observed in basophilic leukocytes, hundreds of which have been examined supravitally for prolonged periods. It is also important to note that none of the other types of white blood cells appeared to be affected by doses of 48/80 which rabbits are able to survive. Rarely, we have observed the appearance of a few red blood cell ghosts in supravital spreads.

Numerous attempts have been made to reproduce this degranulation *in vitro*, by injecting small quantities of 48/80 into blood in siliconized tubes. These trials have never been successful. With the rare observation of red cell ghosts, we were led to the trial *in vitro* and *in vivo* of haemolytic enzymes, feeling that there may be an enzymic mechanism for the degranulation. In the rabbit, intravenous injection of rattlesnake (*Crotalus atrox*) venom, 1 mg/kg, led to the complete disappearance of basophils in the counting chamber. Instances of degranulation were observed in the supravital spread made after 5 min, while there was complete absence of basophils in the supravital spread made 15 min after injection of the venom. The rabbit survives this dose. Venom detoxified by heating produced essentially no response in basophil count or morphology. Preliminary findings indicate that venom will produce degranulation of basophils *in vitro* as well as *in vivo*.

DISCUSSION

We believe that this is the first reported observation of degranulation of basophilic

leukocytes in response to a drug. Thus, though these cells have a different stem cell origin from the tissue mast cell,[17] at least in some species of animals they are capable of responding in a similar manner to 48/80, with respect to decrease in numbers and occurrence of degranulation. Boseila[12] suggested that the change in basophil count following 48/80 might be due to a migration of these cells into tissues, where they might substitute for mast cells in organizing oedema. We believe that this probably does not occur, but that the change in count is produced by a disruption of these cells within the blood, so that they are no longer identifiable as basophils. They may in this way participate in the histamine-release in the rabbit. Hogberg and Uvnas[18] have shown that lecithinase A may be responsible for the disruption of the wall of the mast cell granule following 48/80. Our experiments make it seem likely that a similar mechanism may be involved in degranulation of the basophil, although further work will be required to determine if this is the effective enzyme of the *Crotalus atrox* venom which we used. The frequent occurrence of vacuoles in conjunction with the disappearance of granules we interpret as evidence of the release of a large amount of osmotically active material (histamine, heparin?) previously held in another form inside the granule.

It is of interest that colchicine, which destroys mast cells, produces in the rabbit a marked basophilic leukocytosis upon long-continued subcutaneous administration. This phenomenon demands further study, particularly the dose–effect relationship, effect of various routes of administration, mechanism and species specificity. It seems evident that this basophilic leukocytosis has no relationship to histamine-release, since the typical histamine-releaser 48/80 fails to induce a similar response.

Some studies have been started with other histamine-releasers in the rabbit. This will help to determine whether basophil degranulation generally occurs along with the liberation of histamine in this animal or whether the phenomenon is specific for 48/80.

SUMMARY

In the cockerel and in the rabbit there is a rapid fall of basophil count beginning 5 min after injection of 48/80. In the rabbit, degranulation of these cells was observed in the peripheral blood, at a time which suggests that this phenomenon participates in the shock response to 48/80. The degranulation, and the loss of basophils from blood also occurs after the administration of *Crotalus atrox* venom, suggesting an enzymic attack on the wall of the granule. No tolerance is developed to the basophil-depleting action of 48/80. Colchicine, but not 48/80, produces a basophilic leukocytosis after long-continued treatment.

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STIMULATION OF SECRETION AND METABOLISM IN MOUSE PAROTID GLANDS *IN VITRO**

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Abstract—Adrenaline and pilocarpine each stimulate oxygen uptake and amylase secretion by intact mouse parotid glands *in vitro*. The effects of adrenaline and of pilocarpine on oxygen uptake are additive; their effects on amylase secretion do not summate. The presence of a serum globulin fraction in the medium is necessary to demonstrate a stimulation of oxygen consumption by adrenaline; calcium in the medium is necessary to obtain a consistent stimulation of amylase secretion by pilocarpine. The stimulation by adrenaline of oxygen uptake is prevented by dibenamine, by *d*-tartrate or by killing the mice with ether; the stimulation by adrenaline of amylase secretion is not influenced by any of the compounds studied. The stimulation of both oxygen uptake and of amylase secretion by pilocarpine is prevented by atropine or by killing the mice with ether. It is concluded that adrenaline has a "calorigenic" effect unrelated to its action on amylase secretion. The two effects of pilocarpine are more closely related although, under some conditions, e.g. in the absence of calcium in the medium, the one can be observed in the absence of the other.

THE first studies of the stimulation of secretion and metabolism in salivary glands were reported by Barcroft and Piper[1] in 1912. They observed a secretion of saliva and an increase in the oxygen consumption of the gland when the sympathetic nerve supply to the submaxillary gland in the intact cat was stimulated or when adrenaline was injected. Stimulation of the chorda tympani nerve or injection of pilocarpine had a similar effect on the dog submaxillary gland.[2] Barcroft attributed the increased oxygen consumption, which occurred after the period of maximal secretion, to metabolic processes in the gland restoring it to its previous energy level.

Various studies have been reported concerning the stimulation of the secretion of amylase and of the metabolism of glandular tissue *in vitro*; the tissues used have usually been salivary glands and pancreas. Sometimes both secretion and metabolism were stimulated by one agent; sometimes only one process and not the other was stimulated. The result obtained depended on the species, tissue and stimulating agent used. For example, in mouse parotid glands both the oxygen consumption and the secretion of amylase were stimulated by parasympathomimetic agents[3]; this was also the case with rabbit parotid gland slices,[4] with mouse pancreas[5] and with cat pancreas slices.[6] However, only the secretion of amylase and not the oxygen consumption was stimulated by acetylcholine in pigeon pancreas slices[7] and by adrenaline in rabbit parotid gland slices[4, 8] or in mouse parotid glands.[3]

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The object of the experiments to be described in this paper was to find out what, if any, was the relationship between the stimulation of secretion and the stimulation of oxygen consumption in glandular tissue. The mouse parotid gland was chosen as the tissue to be used since it is sufficiently small and thin to be used *in vitro* without slicing. Extrusion of amylase was taken as a measure of secretory activity *in vitro*; this may not necessarily correspond to the secretory activity seen *in vivo* as measured by the volume of saliva secreted.

METHODS

Incubation of the glands. Four male white mice, each weighing between 20 g and 30 g, were used in each experiment; four glands, one from each mouse, served as controls and the remaining four glands served as the experimental tissue to which the stimulating agent was added. Preliminary experiments showed that the oxygen consumption and amylase secretion by the unstimulated glands were the same in the two groups (Table 1).

The glands were incubated in Warburg flasks (volume 15 ml) containing 1·59 ml of Krebs' phosphate Ringer solution (KPR) (medium II, type *A*[9]) and 0·3 ml of 5·6 per cent glucose solution in the main compartment, 0·21 ml of KPR in the side bulb and 0·2 ml of 20 per cent sodium hydroxide solution in the centre well; the 0·21 ml of KPR in the side bulb of the control flasks was replaced by an equal volume of *L*-adrenaline or pilocarpine solution in the experimental flasks. Incubations were carried out in an atmosphere of oxygen and at 37°C; the oxygen consumption of the glands was measured for 60 min after the contents of the side bulb had been added to the main compartment. Immediately after the incubation the glands were carefully removed from each flask and placed in a glass container; the incubation medium was washed into a separate container.

Amylase estimations. The amylase activities of glands and incubation medium were estimated separately. The glands were homogenized in distilled water and the incubation media were diluted in distilled water; the final volume of these samples was 10 ml. Amylase estimations were carried out out by the method of Smith and Roe[10]; this method was modified for use with the salivary gland extracts in that 3 mg of starch and 3 mg of iodine were used in each sample.

Adrenaline assays. Adrenaline was assayed using the isolated, perfused frog heart.

Calculation of results. The amount of oxygen consumed by the glands is expressed as microlitres of oxygen per mg of tissue (wet weight) per hour, i.e. as qO_2. The increase in oxygen uptake caused by the stimulating agent is calculated as microlitres O_2 per mg of tissue (wet weight) per hour by subtracting the control qO_2 from the stimulated qO_2.

Amylase activity is expressed in units of amylase, as defined by Smith and Roe[10]. The total amylase activities in the glands and in the incubation medium at the end of the incubation were calculated separately as units of amylase; the values for glands and incubation medium were added to give the total amylase activity in the whole system. That percentage of the total amylase activity found in the medium is designated "amylase release". The difference between the amylase release in the control glands and the amylase release in the stimulated glands is used as a measure of the stimulation of secretion. For example, about 20 per cent of the total amylase activity was usually found in the incubation medium containing the control glands; if 70 per cent of the

total amylase was found in the incubation medium containing the stimulated glands, the stimulation of amylase secretion would be 70 − 20 = 50 units. This method of calculation was employed in preference to one in which absolute values of amylase activity was used since the activity varied considerably from one group of mice to the next.

Compounds used. L-Adrenaline base (dissolved in 0·1 N hydrochloric acid), *l*-adrenaline-*d*-bitartrate and pilocarpine hydrochloride were used; their concentration is expressed as w/v in terms of the base. All dilutions were made in KPR.

Serum-protein fractions used. Globulin fraction of human plasma was prepared by precipitating citrated human plasma at 0°C by 50 per cent saturation with ammonium sulphate. The precipitate was collected by centrifugation, redissolved in distilled water and dialysed against distilled water at 2°C for 24 hr; the final dilution in terms of the original plasma was 1 in 2·25.

Albumin fraction was prepared by saturating with ammonium sulphate the supernatant from the globulin precipitation. The precipitate was centrifuged down, redissolved in distilled water and dialysed against distilled water at 2°C for 24 hr; the dilution was the same as that of the globulin fraction.

Mouse serum was obtained by allowing mouse blood to clot; the clot was removed by centrifugation.

RESULTS

The action of pilocarpine

Pilocarpine stimulated both oxygen consumption and amylase secretion (Table 1).

TABLE 1. THE STIMULATION BY ADRENALINE AND BY PILOCARPINE OF OXYGEN CONSUMPTION AND AMYLASE SECRETION BY MOUSE PAROTID GLANDS *in vitro*

Stimulating agent	Incubation medium	No. of expts.	Oxygen uptake ($\mu l.O_2$/mg per hr)			Amylase secretion	
			Control	Increase with stimulating agent		Increase with stimulating agent	
			Mean	Mean	±s.e.	Mean	±s.e.
1. None	KPR	6	1·93	+0·04	±0·028	+1·4	±2·49
2. Adrenaline	KPR	11	2·02	+0·13	±0·100	+16	±7·80
3. Adrenaline	KPR+globulin	24	1·74	+0·45	±0·047	+49	±4·48
4. Adrenaline	KPR+albumin	4	1·62	+0·11	±0·100	—	—
5. Pilocarpine	KPR	24	1·42	+0·36	±0·039	+14·5	±4·51
6. Pilocarpine	KPR+calcium	6	1·44	+0·40	±0·085	+57·3	±5·83
7. Adrenaline and pilocarpine	KPR+globulin	6	1·80	+0·85	±0·079	—	—
8. Adrenaline	KPR+globulin +Ca	4	—	—	—	+53·4	±5·63
9. Pilocarpine	KPR+globulin +Ca	4	—	—	—	+72·8	±3·50
10. Adrenaline and pilocarpine	KPR+globulin +Ca	4	—	—	—	+52·3	±8·57

Adrenaline or pilocarpine concentration: 4×10^{-6} (w/v in terms of the base). KPR is calcium-free Krebs' phosphate Ringer solution. The increase in amylase secretion is the difference between that percentage of the total amylase activity released into the medium by the control glands and that percentage released into the medium by the stimulated glands. For the method of calculation of amylase activities see the Methods section of the text. Oxygen uptake is calculated as μl of oxygen per mg of tissue (wet weight) per hour.

The stimulation of amylase secretion by pilocarpine was rather variable when the glands were incubated in a calcium-free medium; in twenty-four experiments with a pilocarpine concentration of 4×10^{-6} a mean stimulation of amylase secretion of 14·5 units was obtained but this stimulation varied from 0 to 65 units. The mean value of 14·5 units was obtained but this stimulation varied from 0 to 65 units. The mean value of 14·5 units is lower than that obtained by Lesser[3] for the stimulation of amylase secretion by pilocarpine; this low result was later found to be due to the use of a calcium-free incubation medium. When the incubation was carried out in a medium containing calcium (medium III[9]) the mean stimulation of amylase secretion by pilocarpine was 57·3 units and there was good stimulation in every experiment. The presence of calcium in the incubation medium did not alter the stimulation of oxygen consumption by pilocarpine (Table 1). In Fig. 1 are shown dose–response curves for the stimulation by pilocarpine of oxygen consumption and amylase secretion by mouse parotid glands.

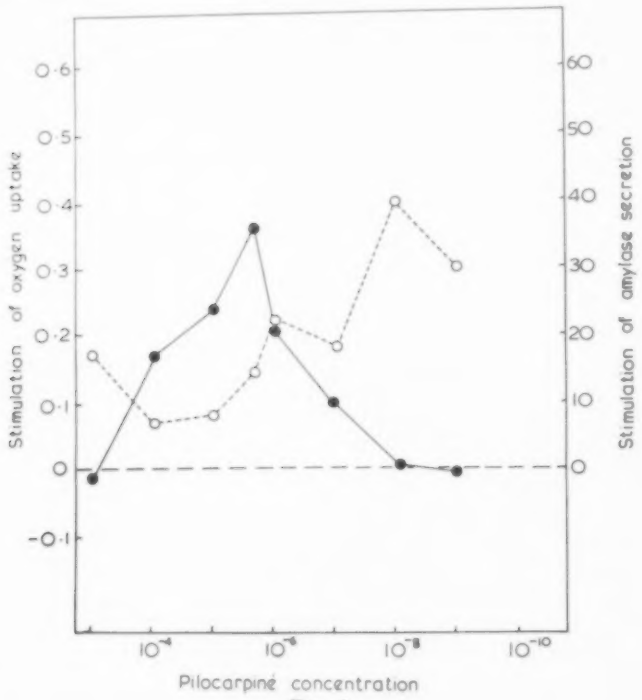

FIG. 1

FIG. 1. Dose-response curves showing the stimulation by pilocarpine of the oxygen consumption and the amylase secretion by mouse parotid glands *in vitro*. Incubation medium: calcium-free KPR (Krebs' phosphate Ringer solution). Pilocarpine concentrations are given as w/v in terms of the base. Each point represents the mean of at least five experiments. ● represent stimulation of oxygen uptake; ○ represent stimulation of amylase secretion. The unit used for the stimulation of oxygen uptake is the difference, in microlitres oxygen per ml of tissue (wet weight) per hour, between the oxygen uptake of the control glands and the oxygen uptake of the stimulated glands. The unit used for the stimulation of amylase secretion is the difference between the percentage of the total amylase activity released into the medium by the control glands and that percentage of the total amylase activity released into the medium by the stimulated glands. For the method of calculation of the actual amylase activities see the Methods section of the text.

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The action of adrenaline

When adrenaline (4×10^{-6}) was added to the parotid glands incubated in a calcium-free medium the stimulation of oxygen consumption was quite variable. In eleven experiments the mean increase in oxygen uptake was 0·13 but the individual results varied from an increase of 0·47 to a decrease of 0·82. This low and variable stimulation of oxygen consumption is similar to that obtained by Lesser[5].

The impression was gained that a better stimulation of oxygen consumption was obtained when more blood was left in or on the glands. Accordingly, a series of experiments was carreid out in which 0·3 ml of mouse serum was added to the incubation medium in the main compartment. In eight experiments a high and consistent stimulation of oxygen consumption was observed. In the next series of experiments a globulin fraction of human plasma was added in place of the serum in order to see whether the protein or some other constituent of the serum might be responsible for the increase in the stimulation by adrenaline; in all twenty-four experiments a consistent stimulation of oxygen consumption was observed. Thus, the addition of human globulin fraction enhanced the stimulation by adrenaline of the oxygen consumption of parotid glands; human albumin fraction did not have this effect (Table 1). In all subsequent experiments in which adrenaline was used as the stimulating agent 0·3

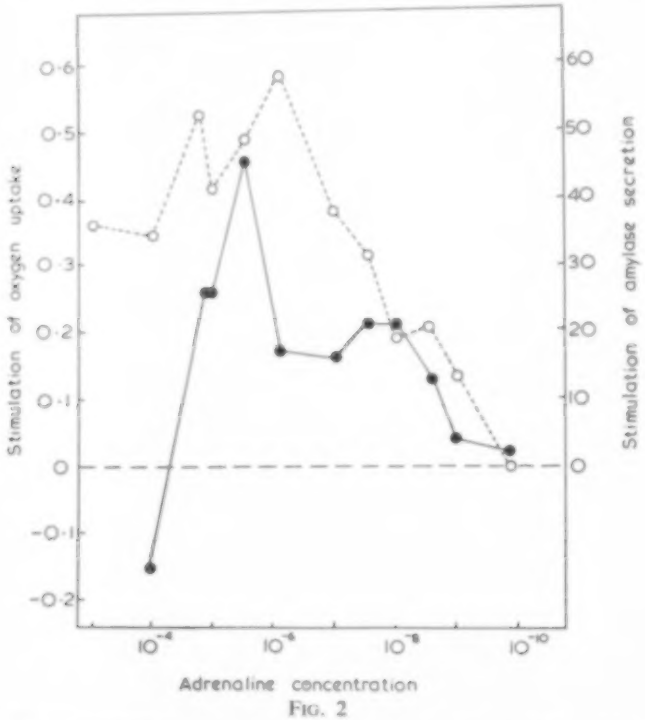

FIG. 2

FIG. 2. Dose-response curves showing the stimulation by adrenaline of the oxygen consumption and the amylase secretion by mouse parotid glands *in vitro*. Incubation medium: calcium-free KPR containing globulin. Adrenaline concentrations are given as w/v in terms of the base. Each point represents the mean of at least five experiments. The units used for the stimulation of oxygen uptake and for the stimulation of amylase secretion are the same as in Fig. 1.

ml of human globulin fraction was added to the incubation medium in the main compartment. In Fig. 2 is shown a dose–response curve for the stimulation by adrenaline of the oxygen consumption of mouse parotid glands incubated in a medium containing globulin.

Adrenaline also stimulated the secretion of amylase by the parotid glands; the addition of globulin to the medium slightly increased this stimulation (Table 1). In Fig. 2 is shown a dose–response curve for the stimulation of amylase secretion by adrenaline.

In four experiments the adrenaline remaining in the flask at the end of the incubation (1 hr) was assayed; of the 2·8 μg added at the start of the incubation 2·04 μg (73 per cent) were recovered after incubation in the absence of globulin and 2·13 μg (76 per cent) after incubation in the presence of globulin.

The action of dibenamine

When dibenamine hydrochloride, 100 mg/kg, was injected subcutaneously into the mice 12 hr before the glands were removed adrenaline did not stimulate the oxygen consumption of the glands; however, it stimulated amylase secretion to about the same extent as in glands from untreated mice (Table 2). The stimulation by pilocarpine of amylase secretion and of oxygen consumption was not altered by injecting the mice with dibenamine (Table 2).

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TABLE 2. THE EFFECTS OF INJECTING THE MICE WITH DIBENAMINE OR ATROPINE AND OF ANAESTHETIZING THE MICE WITH ETHER ON THE SUBSEQUENT STIMULATION BY ADRENALINE OR BY PILOCARPINE OF THE OXYGEN CONSUMPTION AND AMYLASE SECRETION BY THE PAROTID GLANDS *in vitro*

Stimulating agent	Treatment of the mice	No. of expts.	Oxygen uptake (μl.O_2/mg per hr)			Amylase secretion	
			Control	Increase with stimulating agent		Increase with stimulating agent	
			Mean	Mean	±s.e.	Mean	±s.e.
Adrenaline	1. None	24	1·74	+0·45	±0·047	+49	±4·48
	2. Dibenamine	4	2·01	+0·07	±0·069	+33	±7·42
	3. Atropine	2	1·42	+0·43	±0·078	+50	±2·12
	4. Ether	3	1·48	+0·11	±0·040	+53	±7·08
Pilocarpine	1. None	24	1·42	+0·36	±0·039	+14·5	±4·51
	2. Dibenamine	4	1·71	+0·56	±0·082	+15·2	±4·71
	3. Atropine	2	1·77	−0·09	±0·006	−13	±5·32
	4. Ether	10	1·55	−0·003	±0·068	+2·3	±1·58

Adrenaline or pilocarpine concentration: 4×10^{-6} (w/v in terms of the base). Incubation medium: KPR (calcium-free Krebs' phosphate Ringer solution); globulin fraction was added in those experiments in which adrenaline was used. Dibenamine hydrochloride, 100 mg/kg, was injected subcutaneously 12 hr previously. Atropine sulphate, 10 mg/kg, was injected subcuteneously 2 hr previously. Ether anaesthesia: the mice were placed in a jar containing ether until they had stopped breathing. For the calculation of amylase secretion see Table 1 and the Methods section of the text. Oxygen uptake is calculated as μl of oxygen per mg of tissue (wet weight) per hour.

The action of atropine

When atropine sulphate, 10 mg/kg, was injected subcutaneously into the mice 2 hr before the glands were removed, pilocarpine failed to stimulate either the amylase

secretion or the oxygen consumption of these glands (Table 2). Atropine had no effect on the stimulation by adrenaline of either oxygen consumption or amylase secretion (Table 2).

The effect of ether anaesthesia

Pilocarpine did not stimulate either oxygen consumption or amylase secretion in parotid glands from mice which had been killed with ether (Table 2). The resting oxygen consumption of these glands was no different from that of glands from mice killed by breaking their necks.

Adrenaline failed to stimulate the oxygen consumption of glands from mice killed with ether although it did stimulate amylase secretion by these glands to about the same extent as it did in normal glands (Table 2).

The action of tartrate

L-Adrenaline-*d*-bitartrate failed to stimulate the oxygen consumption of the parotid glands although it did stimulate amylase secretion. The addition of *d*-tartrate (4×10^{-6}) to the incubation medium prevented the stimulation by adrenaline (4×10^{-6}) of oxygen consumption but had no effect on the stimulation by adrenaline of amylase secretion. The action of pilocarpine on either process was not altered by the addition of tartrate to the medium. Tartrate alone had no effect on the oxygen consumption or amylase secretion of the glands. The experiments with tartrate are more fully described and discussed elsewhere.[11]

The actions of adrenaline and pilocarpine together

Six experiments were carried out in which adrenaline and pilocarpine (each 4×10^{-6}) were added together to the glands; the concentration used of either would be expected to produce a maximal stimulation of oxygen consumption if the compounds were used separately. The increase in oxygen consumption observed was 0·85 which is approximately the sum of the stimulations observed when either drug was added alone, with adrenaline 0·45 and with pilocarpine 0·36 (Table 1). The actions of adrenaline and pilocarpine on amylase secretion did not summate. In four experiments, in which the glands were incubated in a calcium-containing medium, the stimulation obtained with adrenaline and pilocarpine together amounted to 52·3 units whereas the stimulation obtained with adrenaline alone was 53·4 units and that with pilocarpine was 72·8 nuits (Table 1).

The action of adrenaline under anaerobic conditions

In four experiments adrenaline was added to glands incubated in an atmosphere of nitrogen. Under these conditions adrenaline did not stimulate amylase secretion.

DISCUSSION

Adrenaline and pilocarpine can stimulate both the oxygen consumption and amylase secretion by mouse parotid glands *in vitro*. That pilocarpine has this action was shown previously by Lesser[3], but he failed to demonstrate a stimulation of oxygen consumption by adrenaline, although he did observe a stimulation of amylase secretion by adrenaline. The experiments described in this paper indicate that under suitable conditions adrenaline can stimulate the oxygen consumption of parotid glands.

The presence of a globulin fraction of serum is necessary to demonstrate this stimulation. The mechanism of action of the globulin is not known. It is possible that it acts by influencing the permeability of the gland cells. The presence of serum protein in the incubation medium prevents the usual uptake of sodium by the isolated rat diaphragm incubated in a saline medium[12] and permits maintained oxygen uptake of dog nerve *in vitro* for long periods.[13] It also seemed possible that the added globulin might act by preserving the adrenaline from autoxidation;[14] since about 70 per cent of the added adrenaline was recovered whether it was incubated in the presence or absence of globulin, it is unlikely that the globulin acted by preserving the adrenaline.

The two effects of adrenaline on the parotid glands, i.e. the stimulation of oxygen consumption and the stimulation of amylase secretion, can be separated. The stimulation of oxygen consumption by adrenaline was prevented by injecting the mice with dibenamine, by adding tartrate to the incubation medium, or by killing the mice with ether before removing the glands; the stimulation of amylase secretion was not affected by any of these procedures. Further evidence for the independence of these two effects comes from a comparison of the dose–response curves (Fig. 2); concentrations of adrenaline can be found which stimulate amylase secretion (10^{-3}, 10^{-4}) while actually depressing the oxygen consumption of the glands. It seems clear that the increase in oxygen consumption caused by adrenaline above the resting oxygen consumption of the glands is not necessary for the stimulation of amylase secretion to occur. However, some oxidative processes must be required for the stimulation of amylase secretion since no stimulation was observed when the glands were incubated in an atmosphere of nitrogen. It is known that the stimulation by acetylcholine of amylase secretion by slices of pigeon pancreas is prevented by anaerobic conditions.[15]

Adrenaline is known to have a calorigenic effect both *in vivo* and *in vitro* (reviews by Griffith[16] and Ellis[17]). By calorigenic effect is meant the action of adrenaline to increase the oxygen consumption of a tissue apart altogether from any other activities of that tissue also stimulated by adrenaline, e.g. contraction or secretion, which may also increase the oxygen consumption indirectly. Since the increase in oxygen consumption caused by adrenaline in the mouse parotid gland *in vitro* is not related to the secretion of amylase it is probable that it is a manifestation of the calorigenic effect of adrenaline. Further studies on the calorigenic effect of adrenaline *in vitro* will be reported in a later paper.

There are some indications that the stimulation of oxygen consumption by pilocarpine is not related to the stimulation of amylase secretion. The presence of calcium in the medium increased the stimulation by pilocarpine of amylase secretion about fourfold whereas it had no effect on the stimulation of oxygen consumption; the dose–response curves for the stimulation of the two processes do not coincide. In general, however, procedures which prevented the one stimulation also prevented the other; these procedures include the injection of atropine into the mice or anaesthetizing the mice with ether. It would be more reasonable to suppose that the stimulation of oxygen consumption by pilocarpine is a part of the mechanism for the stimulation of amylase secretion even although it is not directly dependent on it.

The fact that the actions of adrenaline and pilocarpine on the oxygen consumption summate might be explained as a direct stimulation of the cells by adrenaline while the effect of pilocarpine might be a by-product of the secretory process. The actions of these two agents on amylase secretion do not summate; since they are probably

both acting on the same secretory process it is perhaps not surprising that competition is seen when they are added together. The initial stages by which each activates the secretory process must be quite different since the action of pilocarpine is prevented by atropine or by killing the mice with ether, whereas the action of adrenaline is not affected by any of the substances studied. In the experiments of Strömblad,[18] in which slices of cat submaxillary gland were used, no summation of the actions of adrenaline and pilocarpine on oxygen consumption was observed; this was taken as support for the theory of dual innervation of the cells in this gland. The summation of these two actions in the mouse parotid gland *in vitro* does not exclude a dual innervation of the cells of this gland. This summation is interpreted as being due to the different ways in which adrenaline and pilocarpine act on these cells to cause an increase in oxygen consumption.

SUMMARY

(1) Adrenaline and pilocarpine can stimulate both oxygen consumption and amylase secretion by mouse parotid glands *in vitro*.

(2) The presence of a serum globulin fraction in the incubation medium is necessary to demonstrate a stimulation of oxygen consumption by adrenaline. The presence of calcium in the medium is necessary to obtain a consistent stimulation of amylase secretion by pilocarpine.

(3) The stimulation by adrenaline of oxygen consumption is prevented by dibenamine, by *d*-tartrate and by killing the mice with ether; the stimulation of amylase secretion is not influenced by any of the substances studied. The stimulation of both oxygen consumption and amylase secretion by pilocarpine is prevented by atropine or by killing the mice with ether.

(4) The relation between the stimulation of amylase secretion and the stimulation of oxygen consumption is discussed. It is concluded that adrenaline has a calorigenic effect unrelated to its action on amylase secretion.

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THE TUMOUR GROWTH INHIBITORY ACTIVITY OF SOME SULPHUR MUSTARD GAS DERIVATIVES

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Abstract—Two oxidation products of sulphur mustard gas (III), the sulphoxide (IV) and the sulphone (V) have been tested as tumour growth inhibitors—the former shows activity of a low order. The sulphilimine (VIII) derived from (III) is an effective tumour growth inhibitor which is converted into the divinyl compound (IX) in buffered solution. A novel S→ N allylic rearrangement has been encountered in studies of the related sulphilimine of diallyl sulphide.

INTRODUCTION

The concept of latent activity

Alkylating agents such as sulphur and nitrogen mustard gas derivatives, ethylene imines, epoxides and methanesulphonates are toxic to rapidly dividing cells and many of the compounds will inhibit the growth of transplanted animal tumours. One disadvantage of these agents as far as the chemotherapy of cancer is concerned is their relative lack of specificity towards neoplastic tissue; other rapidly proliferating cells such as those in the bone marrow, gastric mucosa, and gonadal tissue are also adversely affected.

For some years now[1] we have been studying alkylating compounds which would not themselves be expected to be active but which could be converted *in vivo* into active agents. It is to be hoped that the less active precursor, which has become known as the "transport form" would be selectively activated at discrete sites and that a measure of specificity of action would result. A similar approach to this problem of selectivity has been made by other groups of workers.[2]

EXPERIMENTAL

All melting points, which are corrected, were taken in sealed capillaries.

Sulphilimine of di-2-chloroethylsulphide

A mixture of thiodiglycol (40 ml) and concentrated hydrochloric acid (200 ml) was heated on steam for 2 hr. The initially clear solution soon became cloudy and a lower oily layer formed. The cooled mixture was extracted with ether and after washing with water the ether layer was dried over calcium chloride. A solution of chloramine-T (40 g) in water (200 ml) was added to a portion of the di-2-chloroethyl sulphide (20 ml) obtained by evaporating the above extract. Vigorous shaking soon led to the separation of colourless crystals; these were collected by filtration and washed with water and light petroleum. After recrystallisation from ethanol or chloroform-light petro-

leum (b.p. 40–60°C) the *sulphilimine* (VIII) had m.p. 143–144°C (Mann and Pope[4] give m.p. 144·5°C).

When 0·1 N sodium hydroxide was added at room temperature to a solution of this sulphilimine (164 mg, 0·0005 mole) in aqueous acetone (1 : 1, v/v, 50 ml) containing phenolphthalein as indicator 10·1 ml were required to render the solution permanently alkaline. Addition of sodium thiosulphate to this titrated solution caused it to become more alkaline and then 10·0 ml of 0·1 N hydrochloric acid were required to neutralize the solution.

After heating a solution of either the sulphilimine (VIII) or the sulphone (IV) (0·0005 mole) in aqueous acetone (1 : 1, v/v, 50 ml) under reflux for 30 min only 0·05 ml of 0·1 N sodium hydroxide were required to render the solution alkaline. This represents a hydrolysis rate of under ½ per cent under standard conditions (cf. Ross[8]).

Sulphilimine of divinyl sulphide (IX)

To a solution of the above sulphilimine (VIII) (6 g) in acetone (200 ml) was added slowly with vigorous stirring aqueous sodium hydroxide (N, 36 ml). Removal of the acetone at room temperature under reduced pressure caused the precipitation of a white solid which was washed with a little iced water and then dried over calcium chloride in a vacuum desiccator. The *sulphilimine of divinyl sulphide* (IX) which was very soluble in acetone but sparingly soluble in benzene, *cyclo*hexane, and light petroleum crystallized with difficulty but the following procedure was successful. An acetone solution was filtered through a small pad of activated alumina and *cyclo*hexane was added dropwise until a slight turbidity formed; crystals slowly separated and as the solution cleared a further drop of *cyclo*hexane was added. This process was continued until the addition of *cyclo*hexane caused no cloudiness: rapid addition caused an oily precipitate to be formed. The product was obtained as pearly plates, m.p. 94–96°C (Found: C, 51·4; H, 5·4; N, 5·3; S, 24·5. $C_{11}H_{13}O_2NS_2$ requires C, 51·7; H, 5·1; N, 5·5; S, 25·1%).

The alkalinity developed on adding an excess of aqueous sodium thiosulphate to a solution of 75 mg of (IX) in 50 per cent aqueous acetone required 6·0 ml of 0·1 N hydrochloric acid for titration (this corresponds to an equivalent of 127; the required value is 255/2 or 127·5).

Sulphilimine of diallyl sulphide (X)

A solution of chloramine-T (10 g) in water (70 ml) was shaken with diallyl sulphide (5 ml) and the product was isolated as above. Crystallization was difficult but was accomplished by dissolution in benzene followed by the gradual addition of light petroleum. The *sulphilimine* (X) formed glistening plates, m.p. 71°C (Found: C, 54·9; H, 5·9; N, 4·9; S, 21·7. $C_{13}H_{17}O_2NS_2$ requires C, 55·1; H, 6·0; N, 5·0; S, 22·6%). A solution of (X) in aqueous acetone developed alkalinity when aqueous thiosulphate was added suggesting that some (XI) had been formed—a quantitative amount of alkali was not liberated in this case.

On keeping the crystals in a desiccator for 24 hr a clear colourless liquid had formed. A solution of this liquid in benzene was passed through a column of activated alumina and elution proceeded with fresh benzene. Early eluates gave a small amount of oil which developed alkalinity with thiosulphate but later washings gave a solid, m.p. 63–64°C, prismatic needles from ether-pentane. Analysis indicated that this compound

was N-allyl-*p*-toluenesulphonamide (Found: C, 57·2; H, 6·3; N, 6·9; S, 15·2. $C_{10}H_{13}O_2NS$ requires C, 57·0; H, 6·2; N, 6·7; S, 15·2%) and the melting point was not depressed by admixture with an authentic specimen, m.p. 62–64°C, prepared by the action of *p*-toluenesulphonyl chloride on allylamine in pyridine solution.

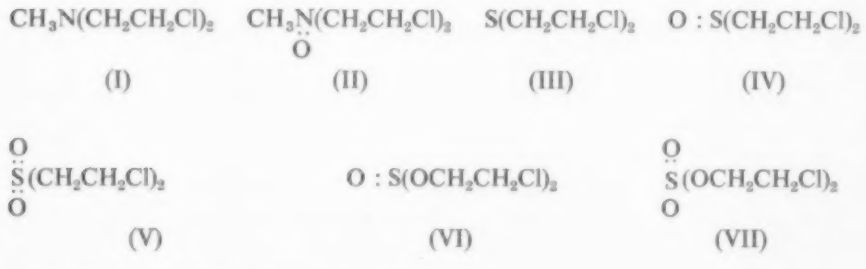
Oxidation of the sulphilimine of di-2-chloroethyl sulphide

To a solution of the sulphilimine (3 g) in glacial acetic acid (30 ml) was added slowly powdered chromium trioxide (5 g). The mixture was heated on steam when a vigorous reaction occurred. After 20 min heating an excess of methanol was added and the mixture was diluted with water. A dried ether extract yielded a solid which crystallized from ether-pentane as flattened needles, m.p. 55–56°C, undepressed by admixture with di-2-chloroethyl sulphone.

RESULTS AND DISCUSSION

Oxides of sulphur mustard gas

Di-2-chloroethylmethylamine (I, nitrogen mustard gas, HN2) is an effective cytotoxic agent and special advantage has been claimed for the use of its N-oxide (II, nitromin)[3]. It is possible that nitromin can be regarded as a less active transport form in the sense referred to above and that it is converted back into HN2 by *in vivo* reduction. As an extension of this particular approach it occurred to us that similar oxidation products of sulphur mustard gas (III) would be worth investigating. Of the two oxides of di-2-chloroethyl sulphide the sulphoxide (IV) shows activity against the transplanted Walker rat carcinoma whereas the sulphone (V) is inactive at a dose of 5 mg per rat given daily in arachis oil (intraperitoneal injection) for 10 days after implant. In an experiment using the sulphoxide (IV) the average tumour weight in a control series of animals 13 days after implant was 58 g whereas in a parallel series of animals which had received intraperitoneal injections of 5 mg of drug in arachis oil per 200 g rat per day for 10 days after implant the average tumour weight on the thirteenth day was 8·8 g (control/treated weights = $C/T = 6{\cdot}6$) A single injection of 10 mg of (IV) on the day following implant gave a C/T ratio of 3·25. The related di-2-chloroethyl sulphite (VI) and sulphate (VII) were ineffective at single doses of 20 mg and 5 mg per rat, respectively.

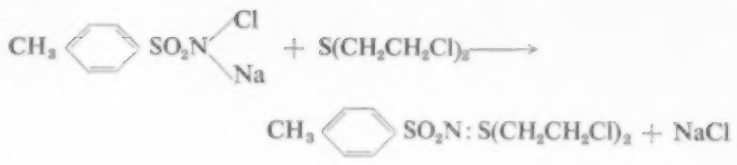


(I) (II) (III) (IV)

(V) (VI) (VII)

The sulphilimine of di-2-chloroethyl sulphide

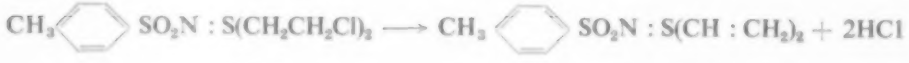
Another compound which might be converted into sulphur mustard gas *in vivo* is the sulphilimine derivative (VIII) which is formed by the action of chloramine-T on the sulphide[4] thus:



(VIII)

A reversal of this formative reaction or reductive fission of the —N : S— linkage would reproduce di-2-chloroethyl sulphide *in situ*. The sulphilimine (VIII, code no. CB 1574) is an effective inhibitor of the transplanted Walker rat carcinoma, a single injection of 10 mg per rat in the standard test gave a *C/T* ratio of 6·8 while ten daily injections of 5 mg per rat starting on the day following implantation gave a *C/T* ratio of 12. A single intraperitoneal injection of 20 mg per rat of CB 1574 in arachis oil prolonged the life of rats inoculated 3 days earlier with a Furth leukaemia cell suspension from 10–13 days to 20–30 days.

On studying the reactions of the sulphilimine (VIII) in aqueous solution it was found that it resembled the sulphone (V)[5] in readily yielding a divinyl compound (IX) in the presence of small amounts of alkali. In fact an aqueous acetone solution of (VIII) could be continuously titrated with decinormal alkali, two equivalents of acid being liberated:



(VIII) (IX)

Reactivity of (VIII) *and* (IX) *towards thiol groups*

A compound structure (IX) would be expected to react readily with highly nucleophilic groups such as thiols and sulphur containing anions and it was, indeed, possible to titrate the divinyl compound quantitatively with alkali in the presence of thiosulphate—a method developed for the estimation of another type of alkylating agent, the epoxide group.[6]

Compounds which react readily with thiol groups have not proved to be effective inhibitors of the Walker rat carcinoma,[7] but this could conceivably be due to the high reactivity of such agents, e.g. iodoacetates, which would be captured before they could diffuse to interact with essential sites in tumours. It has been felt for some time that it would be of interest to test potent thiol reactors with "latent activity", that is compounds which could be transported in a relatively unreactive form but which could be slowly transformed into a reactive agent. The sulphilimine (VIII) may well be such a compound for it is not reactive in unbuffered solution—less than 1 per cent being hydrolyzed when heated for ½ hr in aqueous acetone—but in buffered solution it is gradually transformed into the reactive divinyl compound (IX). Administration of the divinyl compound itself at a single dose of 10 mg per rat gives a *C/T* ratio of 2·3 in the standard test. This low activity would have been expected if most of the agent was being lost by irrelevant reactions which occurred before the compound could have become widely distributed.

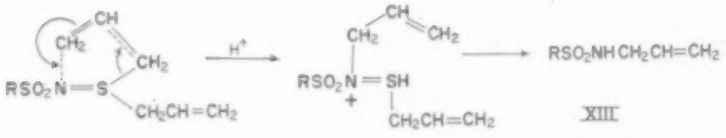
Allylic rearrangement in the sulphilimine of diallyl sulphide

Similar latent activity might be anticipated in the case of the sulphilimine of diallyl sulphide (X) which would have been expected to rearrange by double bond migration into the conjugated and hence activated position as shown in (XI).

(X)

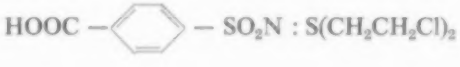
(XI)

The sulphilimine (X) was obtained as a crystalline compound which did not inhibit the Walker rat carcinoma at a dose of 10 mg per rat (single dose administered intraperitoneally in arachis oil). It was shown, however, that the compound was unstable for within 24 hr of isolation the crystals change into a clear liquid from which *N*-allyl *p*-toluenesulphonamide (XIII) has been isolated. The mechanism of this novel $S \longrightarrow N$ allylic rearrangement has not been fully worked out but by analogy with other allyl group migrations the quaternary compound (XII) is probably an intermediate product.



X XII (R = p-tolyl)

The instability of the sulphilimine (X) clearly precludes its transformation into appreciable amounts of the activated homologue (XI) of the divinyl derivative and so its biological ineffectiveness is not unexpected. An attempt to prepare a more water soluble sulphilimine derivative by the oxidation of (VIII) to the carboxy-compound (XIV) led to the formation of the sulphone (V).



(XIV)

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FURTHER STUDIES ON THE INHIBITION OF NUCLEIC ACID BIOSYNTHESIS BY AZATHYMIDINE AND BY DEOXYADENOSINE*

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(*Received* 25 *February* 1959)

Abstract—Azathymidine, an analogue of thymidine, inhibited reversibly the utilization of radioactive thymidine for the biosynthesis of DNA-thymine of Ehrlich ascites tumour cells *in vitro*. The thymidine analogue also inhibited the utilization of radioactive adenine for the biosynthesis of DNA-adenine, but an excess of thymidine did not completely prevent this inhibition. Deoxyadenosine, but not adenosine, adenine or deoxyguanosine, inhibited the utilization of formate and to a lesser extent that of thymidine for the biosynthesis of DNA-thymine.

AZATHYMIDINE, the deoxyribonucleoside of 6-methyl-*asym*-triazine-3 : 5(2H : 4H)-dione, has been shown previously to be an effective inhibitor of the synthesis *de novo* of deoxyribonucleic acid (DNA)-thymine from formate-^{14}C by mouse Ehrlich ascites tumour cells† *in vitro*.[1] Similar results have been obtained with several mouse leukaemia cells *in vitro*: L-4946; L-1210, azaguanine-dependent strain; and both the azaserine-sensitive and -resistant plasma cell leukaemias.[2]

In an attempt to establish the inhibitory specificity of this analogue of thymidine, a study has been made of the effect of several naturally-occurring deoxyribonucleosides on the utilization of formate-^{14}C for the biosynthesis of DNA-thymine of Ehrlich ascites tumour cells. Whereas deoxyuridine and deoxycytidine[1, 3] increased the utilization of formate-^{14}C for the biosynthesis of DNA-thymine, deoxyadenosine markedly inhibited this reaction (Table 1), an effect which could not be duplicated with deoxyguanosine. This inhibitory effect of deoxyadenosine was not exhibited by adenosine or adenine. Klenow and Langer[4] recently obtained similar results independently with the adenine derivatives and, in addition, demonstrated that deoxyadenosine did not exert its adverse effect on the biosynthesis of DNA by a block in the formation of the thymine-containing precursors of DNA. The inhibition of the utilization of formate for the biosynthesis of DNA by deoxyadenosine was about ten times greater than that produced by an equimolar concentration of the thymidine analogue, azathymidine (Table 1). At a level of deoxyadenosine (0·4 mM) which exerted a marked inhibition of the utilization of formate for the biosynthesis of DNA-thymine, an insignificant

* This investigation was supported by a grant (CY-3076) from the National Institutes of Health, United States Public Health Service.

† Appreciation is expressed to Dr. J. J. Jaffe for his advice and for making the mouse tumours readily available.

TABLE 1. EFFECT OF AZATHYMIDINE AND VARIOUS PURINE DERIVATIVES ON THE UTILIZATION OF FORMATE-^{14}C FOR THE BIOSYNTHESIS *in vitro* OF DNA-THYMINE OF MOUSE EHRLICH ASCITES TUMOUR CELLS*

Compound added (μ moles)		Relative specific activity of DNA-thymine
None (control)		1·00†
Azathymidine	1	0·72
Azathymidine	10	0·13
Deoxyadenosine	1	0·26
Deoxyadenosine	10	0·00
Adenosine	10	1·14
Adenine	10	1·18
Deoxyguanosine	1	0·87
Deoxyguanosine	10	0·91

* Specific activity of the control was 10,200 counts/min per μmole thymine.

† The reaction mixtures consisted of 0·25 ml of packed cells, 5 μmoles (5 μc) of formate-^{14}C, pig serum 0·2 ml; a modified Chambers' solution was added to give a final volume of 2·5 ml. The incubations were conducted in 20 ml beakers, in duplicate, in a Dubnoff metabolic shaker at 37°C under air, and agitated at 90 c/min for 4 hr. The isolation of DNA-thymine has been described previously.[1]

inhibition was exerted on the utilization of pre-formed thymidine (Table 2). However, a higher level of deoxyadenosine (4·0 mM) exerted a strong inhibitory effect; a similar result was not obtained with this level of deoxyguanosine.

TABLE 2. THE EFFECT OF DEOXYADENOSINE AND DEOXYGUANOSINE ON THE UTILIZATION OF RADIOACTIVE THYMIDINE FOR THE BIOSYNTHESIS OF DNA-THYMINE OF ERHLICH ASCITES TUMOUR CELLS *in vitro**

Nucleoside added (μ moles)		Specific activity of DNA-thymine
None (control)		13,200
Deoxyadenosine	1	11,300
Deoxyadenosine	10	2700
Deoxyguanosine	10	12,800

* Details of the incubation mixture are described in Table 1. Each beaker contained methyl labelled thymidine-^{14}C (0·01 μmole).

Hall and Haselkorn[5] demonstrated that a thousand-fold excess of azathymidine inhibited by 50 per cent the utilization of thymidine-^{14}C for the biosynthesis of DNA-thymine of rabbit bone-marrow cells *in vitro*. Similar results were observed with Ehrlich ascites tumour cells *in vitro*[6] (Table 3). These results indicate clearly that under these conditions azathymidine is a very inefficient inhibitor of the utilization of thymidine for the biosynthesis of DNA. This probably accounts for the relative ineffectiveness of azathymidine as a chemotherapeutic agent,* since only minute

* Personal communication from Dr. J. J. Jaffe of this department.

amounts of thymidine, derived from exogenous sources or from the degradation of cellular DNA, would be required to prevent the inhibition of the utilization of thymidine by its analogue.

TABLE 3. THE EFFECT OF AZATHYMIDINE ON THE UTILIZATION OF RADIOACTIVE THYMIDINE FOR THE BIOSYNTHESIS OF DNA-THYMINE OF EHRLICH ASCITES TUMOUR CELLS *in vitro**

Concentration of thymidine-^{14}C (μmoles)	Specific activity of DNA-thymine	
	No additions (control counts/min per μmole)	Plus azathymidine (20 μmoles counts/min per μmole)
1·0†	5500	5010
0·10‡	19,800	21,600
0·01‡	13,200	3650

* Details of the incubation mixture are described in Table 1.
† Specific activity of ^{14}C-methyl-labelled thymidine : 1×10^6 counts/min per μmole.
‡ Specific activity of ^{14}C-methyl-labelled thymidine : 5×10^6 counts/min per μmole.

That the inhibition of the utilization of thymidine caused by high concentrations of azathymidine resulted in a reduction in the synthesis of DNA was shown through the concurrent use of adenine-^{14}C (Table 4). It will be seen that a marked inhibition

TABLE 4. THE EFFECT OF AZATHYMIDINE AND THYMIDINE ON THE UTILIZATION OF ADENINE-8-^{14}C FOR THE BIOSYNTHESIS OF RNA- AND DNA-ADENINE OF EHRLICH ASCITES TUMOUR CELLS *in vitro**

Concentration of thymidine (μmoles)	RNA-adenine		DNA-adenine	
	No additions (control counts/min per μmole)	Plus azathymidine (10 μmoles counts/min per μmole)	No additions (control counts/min per μmole)	Plus azathymidine (10 μmoles counts/min per μmole)
0·0	32,800	26,000	3860	880
0·10	48,000	37,000	5000	2810
10·0	46,500	39,000	5570	2820

* Details of the incubation mixture are described in Table 1. Each beaker contained adenine-8-^{14}C, (5 μmoles, 5 μc) and all components of the reaction mixtures were added prior to incubation.

(78–98 per cent) of the utilization of adenine for the biosynthesis of DNA-adenine was observed. This inhibition by azathymidine (AzTdR) was partially prevented by thymidine (TdR) at a ratio of 1 : 100 (TdR : AzTdR); however, even at a ratio of 1 : 1 the degree of interference was not altered to any greater extent. This latter result was not anticipated in view of the complete reversibility of the inhibition by

azathymidine of the utilization of thymidine for the biosynthesis of DNA thymine in this system (Table 3). A small increase (40 per cent) in the extent of the incorporation of radioactive adenine into RNA-adenine was observed in the absence of azathymidine and the presence of thymidine.

DISCUSSION

There are many reasons why a compound, which has been designed successfully to be an analogue of a metabolite in microbial systems, fails to be effective in mammalian systems. Azathymine[7] and its deoxyriboside, azathymidine,[8] have been shown, to be competitive antagonists of the utilization of thymine or thymidine for various microbial systems. Radioactive azathymine was incorporated into the DNA of *Streptococcus faecalis*; however, there did not appear to be a relationship between the extent of incorporation of the unnatural base into the DNA and the lethal effect or inhibition of the growth of this organism.[9] No incorporation of radioactive azathymine into the DNA of regenerating liver of mice was observed *in vivo*,[2] whereas under these conditions a very small incorporation of the corresponding normal metabolite, thymine, did occur. These results were not unexpected since the corresponding deoxyribosides would be the more probable compounds to be incorporated. Thus, Reichard[10] has shown that thymidine was utilized for the biosynthesis of mammalian DNA-thymine to a much greater extent than thymine. Similarly, iododeoxyuridine,[11] has been demonstrated also to be incorporated into mammalian DNA *in vivo*[12] and also under the conditions of cell culture.[13, 14] In order to demonstrate the probable utilization of azathymidine for the biosynthesis of mammalian DNA, radioactive azathymidine would be required. It would be anticipated that only traces of azathymidine would be incorporated into mammalian DNA as compared to that observed with iododeoxyuridine, since the 50 per cent molar inhibition index of iododeoxyuridine for the utilization of thymidine in the biosynthesis of DNA-thymine of Ehrlich ascites tumour cells *in vitro* is 6·0,[2] whereas that of azathymidine is roughly 1000.[5]

Thus, two analogues of thymidine, namely azathymidine[15] and iododeoxyuridine,[11] designed to be antagonists of thymidine, have exhibited such properties; however, under the conditions of their examination in mice, only iododeoxyuridine has exerted marked anti-tumour activity. There is very little difference in the stereochemistry of thymidine and these two analogues, but a marked difference was observed in their pK_a values: thymidine, 9·8; iododeoxyuridine, 8·25; and azathymidine, 7·4. Hence, we see that a more marked disturbance in the physical chemical properties of thymidine resulted from the replacement of the carbon and hydrogen in position-6 of the pyrimidine ring by a nitrogen atom,* than by the replacement of the methyl group of thymidine by an iodine atom. That the relatively inefficient inhibition by azathymidine of the utilization of thymidine for the biosynthesis of DNA, in contrast to that caused by iododeoxyuridine, is a result of the more than a hundred-fold greater dissociation of the "aza" compound as a weak acid than is the case with the normal metabolite, is an attractive hypothesis. If this should indeed be a factor, then it would be advisable to attempt the synthesis of analogues with pK_a values which are reasonably close to

* According to the older numbering system applied to pyrimidines, azathymine would be referred to as 4-azathymine; however, in the system employed by *Chemical Abstracts* the proper name (as a pyrimidine) is 6-azathymine.

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that of the corresponding metabolite. This difference in pK_a value may be reflected by: (a) differences in the affinities of the antimetabolites for the sensitive enzyme (*s*); (b) differences in the ability of the cell to convert the analogues to the appropriate activated derivatives; and (c) an inability to activate the analogue at a rate sufficient to maintain an effective inhibitory concentration.

The failure of thymidine to prevent completely the inhibition by azathymidine of the utilization of adenine for the biosynthesis of DNA-adenine may indicate an additional site of inhibition in addition to that of the utilization of thymidine or its derivative for the biosynthesis of DNA. That azathymidine exerts an inhibitory effect in addition to that related to the biosynthesis of nucleic acid was indicated by the lack of a relationship between the incorporation of azathymine into the DNA of *S. faecalis* and inhibition of growth.[9] Thymidine or its phosphorylated derivative may prove to have physiological importance in addition to that of being a substrate for the biosynthesis of DNA,[15, 16] but no definite evidence to support this possibility has been presented. Whether the observed inability of thymidine to prevent completely, in the presence of azathymidine, the inhibition of the utilization of adenine for the biosynthesis of DNA-adenine is related to the hypothetical coenzymic role of thymidine will require further investigation.

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INHIBITION OF OXYGEN CONSUMPTION IN HUMAN ERYTHROCYTES BY HEXETIDINE*

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Abstract—Hexetidine was found to inhibit the oxygen consumption of methylene blue-stimulated erythrocytes when inosine or glucose-6-phosphate were substrates. At amounts of inhibitor insufficient to cause extensive lysis of cells, TPN competitively reversed the inhibition; a small effect of TPP was observed. Comparable quantities of inhibitor did not influence the amount of glucose catabolized or of lactate produced by the cells. The exchange of phosphate between plasma and cells was likewise not affected.

HEXETIDINE (1:3-bis-β-ethylhexyl-5-methyl-5-aminohexahydro-pyrimidine, abbreviated BEP†), is a "synthobiotic" drug possessing broad antibacterial and antifungal properties.[1] In a preliminary report,[2] growth of *E. coli-B* was inhibited by hexetidine and completely reversed by thiamine. In spores of *B. cereus var. terminalis* and also a particulate fraction derived from these, the oxidation of pyruvate was inhibited by hexetidine and reversed by thiamine.[3] Another report[4] has indicated that the inhibition by hexetidine of the growth of *Psuedomonas aeruginosa* could be reversed singly and to varying degrees by a number of cofactors, namely pyridoxal phosphate, TPN, TPP and biotin. DNA and RNA were also effective. However, little is yet known of the mode of inhibition of this drug in mammalian tissues.

The formed elements of human blood offer several advantages for the elucidation of biochemical pharmacological mechanisms. The cells comprise several kilograms of tissue which come intimately in contact with administered drugs. Furthermore, they are readily obtained and enumerated, and may be maintained in simulated physiological environments, i.e. in plasma under O_2–CO_2 gas tensions. Metabolically, erythrocytes are restricted mainly to glycolysis and certain aspects of the hexosemonophosphate[5, 6] and pentose phosphate[7] pathways. There is no functioning tricarboxylic acid cycle and little, if any, anabolism of simple substrates to fat or glycogen. Some properties of leukocytes especially suited for inhibition studies will be detailed separately. In these tissues, the biochemical and morphological effects of inhibitors may be evaluated by a variety of well developed chemical and microscopic procedures. Data are presented here with erythrocytes which show hexetidine to inhibit the oxygen-consuming ability in preference to the glycolytic pathway. Competitive inhibition is

* A preliminary report containing part of the data described here was presented to the Division of Medicinal Chemistry, American Chemical Society, April 1957.

† Abbreviations used are: BEP, hexetidine (1:3-bis-β-ethylhexyl-5-methyl-5-aminohexahydro-pyrimidine); R-1-P, ribose-1-phosphate; G-6-P, glucose-6-phosphate; 6-P-G, 6-phosphogluconate; MeB, methylene blue; FMN, flavin mononucleotide; DPN, diphosphopyridinenucleotide; TPN, triphosphopyridinenucleotide; TPP, thiamine pyrophosphate; DNA, desoxyribonucleic acid; RNA, ribonucleic acid.

shown with the coenzymes TPN and TPP on the methylene blue–coupled oxygen consumption using inosine as substrate.

EXPERIMENTAL

Erythrocyte suspensions. Blood from normal male medical students was collected in heparinized, non-wettable plastic bags. The cells were obtained by centrifugation (900 × *g* for 15 min); the supernatant plasma was discarded, and the buffy coat of leukocytes was removed with a cotton swab. The erythrocytes were washed twice with 0·11 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7·4) and resuspended with buffer to a volume fraction of cells (hematocrit) of 0·6 for the oxygen consumption studies. White cell-free whole blood for glycolysis measurements was prepared by resuspending cells with plasma containing substrates to a volume fraction of cells of 0·4.

Haemolysates. Haemolysates were prepared by a threefold freezing and thawing of washed and centrifuged erythrocytes.

BEP. Hexetidine solutions were prepared from the pure liquid (an oil) by adding 0·2 ml to diluted HCl at pH 3·0 in a 50 ml volumetric flask. The resultant clear solution was diluted to volume with 0·15 M NaCl after adjusting the pH to 3·85 just prior to final dilution to 50 ml. The concentration of hexetidine was 10·2 μmoles/ml.

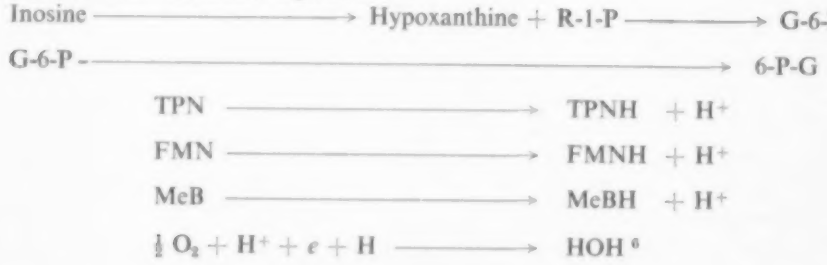
Cofactors. TPN and TPP of commercial purity were dissolved in a minimum volume of 0·15 M NaCl prior to use.

Equilibration technique. Respiration experiments were undertaken in Warburg vessels using standard manometric technique. Glycolytic experiments were carried out in 50 ml silicone-treated flasks in which the erythrocyte suspensions were maintained under one atmosphere of 5 per cent CO_2–95 per cent air and shaken at a rate of 50–60 oscillations per minute. Aliquots were removed at intervals for analysis and deproteinized with $Ba(OH)_2$ (1 : 20) or with 10 per cent trichloroacetic acid (1 : 10).

Methods. Glucose was measured using the Nelson modification of the Somogyi technique,[8] lactate by the Barker–Summerson[9] method, and inorganic phosphate by the method of Fiske and Subbarow[10]. In one experiment with radioactive phosphate, $NaH_2P^{32}O_4$ was added to give a concentration of 0·29 μc/ml of cell suspension. Aliquots were withdrawn at the beginning and at hourly intervals up to 5 hr. Plasma was separated quickly at 2°C and rendered protein-free with 10 per cent trichloroacetic acid (1 : 10). Aliquots (1 ml) of these were taken to dryness and counted with an end window Geiger counter.

RESULTS AND DISCUSSION

Inhibition of oxygen consumption by hexetidine. The inhibition of oxygen consumption produced by hexetidine (BEP) is shown in Fig. 1. The theory of the reaction, using inosine as substrate, is given as:



The phosphorolysis of inosine in erythrocytes yields R-I-P.[11] This is metabolized by an as yet unknown pathway to G-6-P. Dische[12] had previously shown an accumulation of hexosephosphate in adenosine fortified hemolysates of human blood. It is now known[7] that one of the products of the metabolism of ribose-1-phosphate produced from inosine in erythrocyte ghosts is G-6-P. The chief advantage of using inosine in this study was its apparent rapid penetrability into the cells and prompt metabolism to G-6-P, thus permitting use of intact cells for the inhibition study. However, it was later found possible to use G-6-P as substrate, although rates of oxygen uptake were less than for comparable amounts of substrates, suggesting that permeability of phosphorylated hexose is a rate-limiting factor when using intact cells. However, only in the experiments with thiamine pyrophosphate was it found necessary

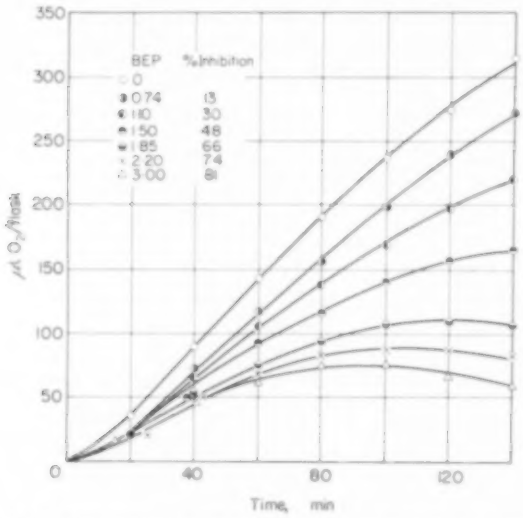


FIG. 1. The effect of BEP on oxidation of inosine by human erythrocytes. The flasks contained 1·7 ml of erythrocyte suspension and 0·2 ml of 0·1 per cent methylene blue in the main chamber. The side arm contained 37·1 μmoles of inosine (0·4 ml) and from 0 to 3 μmoles of BEP. The centre well contained 0·2 ml of 30 per cent KOH. The total volume was 2·9 ml. The columns at the right give the μmoles of BEP and the percentage inhibition.

to use haemolysates in place of intact cells. Figure 1 shows that 1·50 μmoles of BEP (0·000495 M) produced approximately 50 per cent inhibition. The amount of tissue was 1·02 ml of packed erythrocytes containing approximately $1{\cdot}3 \times 10^{10}$ cells. On a numerical basis 50 per cent inhibition was evoked by 1 μg of BEP per 4×10^8 cells. With other blood samples, values from 40–60 per cent were found. Except for the extremes of the concentrations of inhibitor, a linear increase in inhibition was observed with increasing concentration of BEP. In Fig. 1 the slopes of the uptake curves changed continuously at the high concentrations of inhibitor. The initial rates were not as reliable as the net oxygen uptake in 2 hr, since they were less reproducible in the first hour. Preincubation of cells with inhibitor was tried but not employed, as haemolysis (20 per cent) was observed with 3·0 μmoles of inhibitor per flask. Therefore, the inhibitor concentration used was limited to 1·5 μmoles. The surface active nature of hexetidine at the high concentrations probably contributes a component to the

total inhibition observed. This was not further characterized, but was assumed to be not simply linked to cellular metabolic events. It was possible, however, with the unit of oxygen uptake employed and inhibitor concentrations up to 1·48 μmoles per flask to describe the inhibition as competitive for cofactors.

Relative lack of inhibition of glycolysis by hexetidine

Glycolytic experiments were undertaken in which 35·0 ml of erythrocytes were suspended in plasma to a volume fraction of 0·4. The flasks contained heparin (sodium) $NaH_2P^{32}O_4$, and 20 μmoles of glucose per ml of packed cells. Inhibitor was varied from 1·84–5·52 μmoles per ml of packed cells. Controls contained all components except inhibitor. In 4 hr of equilibration at 37°C there were no singificant differences observed in the glucose used or the lactate produced by the cells. Kinetic curves for these, with and without inhibitor, were superimposable and thus showed no inhibition. The stoichiometry of the reaction expressed as a ratio of moles of lactate produced to moles of glucose utilized (theoretically equal to 2·0) was 2·4 in the control flask, 2·4 with 1·84 μmoles of inhibitor per ml of packed cells, and 2·0 with three times as much (5·52 μmoles) inhibitor. In the last instance, the lower value is not significantly different from the others and may have resulted from the moderate haemolysis (24 per cent) caused by the higher concentration of hexetidine. The time variation of inorganic phosphate was the same in all flasks, as was the specific activity of plasma inorganic phosphate. The maintenance of the cells in an approximately steady state of ^{32}P-exchange, and the lack of an increase of inorganic phosphate in the plasma of blood containing inhibitor, attest to the relative lack of influence of hexetidine on the cellular pool of phosphate esters. These observations support the conclusions that hexetidine in concentrations up to three times that used in the oxygen consumption inhibition experiments was without influence on the initial and terminal reactions of glycolysis and on the phosphorylated pool of intermediates between these extremes.

Reversal of inhibition by TPN and TPP

Several cofactors were found to effect the inhibition of hexetidine in the oxygen consuming system. Both TPN and TPP, alone and in combination, were found to reverse partially the inhibited oxygen consumption. TPN was more effective than TPP in systems using either inosine (Table 1) or G-6-P (Table 2) as substrates. The relatively

TABLE 1. ANTAGONISM OF HEXETIDINE BY TPN

Hexetidine (μmoles)	TPN (moles)	Q_{O_2} (μl/flask per hr)
0·00	0·00	150
0·00	0·50	162
0·59	0·50	135
1·18	0·00	48
1·18	0·50	84
1·18	1·0	111
1·18	2·0	122
0·00	1·0*	16

The substrate was 37·1 μmoles of inosine per flask.
* In the absence of substrate, an insignificant consumption of oxygen occurred.

TABLE 2. ANTAGONISM OF HEXETIDINE BY TPN

Hexetidine (μmoles)	TPN (μmoles)	Q_{O_2} (μl/flask per hr)
0·0	0·0	141
0·0	0·5	152
0·0	1·0	169
0·0	1·5	236
1·48	0·0	10
1·48	0·5	36
1·48	1·0	168
1·48	1·5	299
1·48	2·0	370

The substrate was 57 μmoles of G-6-P per flask. Less than 20μl of oxygen were consumed when washed cells without susbtrate were incubated with 1·0 μmole of TPN.

large effect of TPN in the inhibited system, as compared with the uninhibited, is consistent with the postulate that a small percentage of haemolysis disrupted the cell

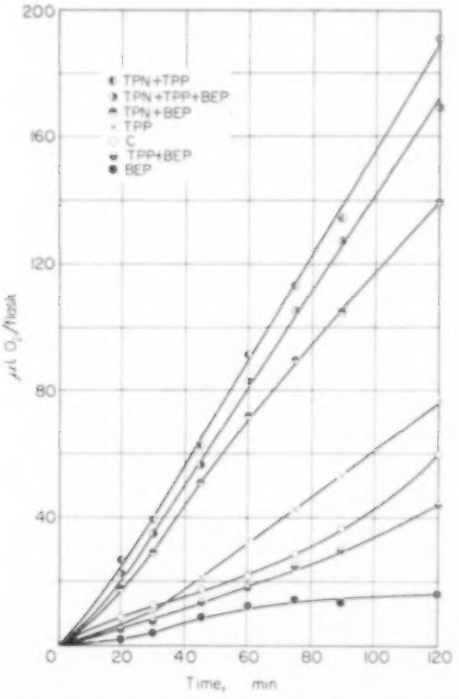


FIG. 2. The reversal of inhibition of oxygen consumption by TPN and TPP in haemolysates of human blood. The flasks contained 1·6 ml of frozen–thawed haemolysate, 0·2 ml of 0·1 per cent methylene blue and either, TPN (0·5 μmoles) or TPP (6·2 μmoles), or both, were present in the centre chamber. The side arm contained 37·1 μmoles (in 0·4 ml) of inosine and BEP (1·48 μmoles). The centre well contained 0·2 ml of 30 per cent KOH; the total volume was 2·9 ml. The curves are labelled for the cofactors and inhibitor composition. *C* is the control (2·9 ml) containing haemolysate, inosine, MeB, and KOH (centre well), but no cofactors or BEP. No significant oxygen uptake was observed in flasks incubated with either TPN or TPP in the absence of inosine.

surfaces sufficiently to facilitate the uptake of TPN by the cells. A progressive diminution of the inhibition was observed as TPN was increased in increments of 0·5 μmoles to 2·0 μmoles per flask. The effect of TPP alone and in combination with TPN is shown in Fig. 2. Haemolysates of erythrocytes were found to be much more dependent on TPN for oxygen consumption and also were much more sensitive to the action of hexetidine. Very little oxygen was consumed in the control reaction without the addition of TPN, probably because of the destruction of endogenous TPN during the preparation of the haemolysate. Conversely, TPP had little effect on the control reaction, but did show a small reversal of the inhibition by hexetidine (30-μl increase). The same magnitude of increase (30 μl) was observed when TPP was added to the flasks in which TPN largely overcame the inhibition by hexetidine, making it apparent that TPP exercised its limited effect through an independent mechanism. It is well known that glucose-6-phosphate dehydrogenase in human erythrocytes has several cofactor requirements, including TPN and a flavin coenzyme.[6] The vitamin constituents of these coenzymes, namely nicotinamide and riboflavin, respectively, were without effect on the inhibition caused by BEP. Participation of TPP even to a small extent implicates hexetidine as a potential inhibitor of transketolase, since TPP is known to be a cofactor for this enzyme.[13] Potentially, cofactor competition between hexetidine and TPP for transketolase in human erythrocytes may diminish the rate of production of glucose-6-phosphate from the pentose of inosine by way of a mechanism producing sedoheptulosephosphate and fructose-6-phosphate.[14] However, the methylene blue-coupled oxygen consumption which is inhibited by hexetidine and reversed to a large extent by TPN is the only reaction in the human erythrocyte which is known to be linked to this cofactor. The relative insensitivity of glycolysis to hexetidine suggests that components of respiratory pathways are specifically inhibited. Support of this view has been derived from observations with human leukocytes which indicate that respiratory dehydrogenases are inhibited by hexetidine and competitively reversed by DPN.*

* In manuscript.

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ANTICHOLINESTERASE ACTIVITIES OF SOME BIS-QUATERNARY AMMONIUM SALTS

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Abstract—A variety of lipophilic-substituted, bis-quaternary ammonium alkane salts are reported to have considerable activity as inhibitors of cholinesterases. With the *in vitro*-enzyme preparations, anticholinesterase activity of these compounds increases with the length of the alkane-linking chain and is favoured by the presence of lipophilic, rigid, nearly planar substituents on the onium nitrogen atoms. The corresponding influences of these structural variations on curarimimetic and related activities are discussed.

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DURING the past several years, there has been prepared and tested in these laboratories a large variety of quaternary ammonium salts with various degrees of activity and specificity as curarimimetics, autonomic blocking agents, hypotensive agents, etc. Most of these pharmacological effects are believed to be related to some aspect of blockade of receptors responsive to acetylcholine. Among the factors which have been considered to be involved in governing selectivity of action of these acetylcholine-receptor blocking agents are the accessibility of the receptor site or area to various quaternaries[1] and the relative distribution of a compound among sites of action and sites of loss*. Factors controlling accessibility include (a) the presence of biological membrances such as the blood–brain barrier, ganglionic sheaths, myelin nerve sheaths and a variety of other cell membrances or surfaces and (b) dimensional, physical limitations imposed by the topography of the environment of the receptor group. Of the acetylcholine receptor sites, probably the most accessible of those located at sessile anatomical loci are at the myoneural junctions susceptible to blockade by curarimimetics. The most accessible of all acetylcholine receptors in the intact animal are probably those situated at the surfaces of extracellular cholinesterases.

Of the variables influencing the distribution of a compound among sites of action and sites of loss, a major one appears to be the relative lipophilic–hydrophilic balance in the compound and the configurational characteristics of the lipophilic moieties. The influence on distribution is particularly important in an intact animal system; in an *in vitro*-measure of anticholinesterase activity with extracellular enzyme preparations distribution becomes a factor only at molecular rather than anatomical levels. The purpose of the present investigation was to determine the effect on cholinesterases of curarimimetic and hypotensive quaternaries embodying some structural modifications which are believed to have an influence on their accessibility and distribution to acetylcholine receptors. Of particular interest were the relative influences of the size

* "Sites of loss" may be considered as sites of adsorption of a compound other than sites of action. Adsorption at sites of loss usually does not produce a measureable response (see also H. Veldstra, *Pharmacol. Rev.* **8**, 342 (1956).

and shape of the lipophilic substituents on reversible cholinesterase inhibitory activities.

EXPERIMENTAL MATERIALS AND METHODS

Most of the compounds used in this study have been described previously. In addition to some common reference agents, the compounds tested included some symmetric, bis-quaternary curarimimetics[2,3] and some unsymmetric, bis-quaternary hypotensive agents[4,5] which embody significant variations in the size and configuration of the terminal lipophilic substituents on the onium nitrogen atoms and in the length of the connecting polymethylene chain. The enzyme preparations used were bovine erythrocyte cholinesterase* ("true cholinesterase") with acetylcholine substrate and horse-serum cholinesterase† ("pseudo-cholinesterases") with acetylcholine or butyrylcholine substrates.

The influence of the compounds on cholinesterase activities was measured as follows. Anticholinesterase activity was determined *in vitro* by standard manometric methods[6] at 37 °C with a gas phase of 95% N_2–5% CO_2. Acetylcholine bromide (0·02 M) was employed as substrate for Winthrop Laboratories bovine erythrocyte acetylcholinesterase (10 units/ml), and either acetylcholine bromide (0·02 M) or butyrylcholine chloride (0·01 M) as substrate for horse-serum cholinesterase (diluted 1:20 with Krebs–Ringer bicarbonate buffer). The inhibitors were added to the main chamber of the Warburg vessels, and substrate was tipped in after a 10 min equilibration period. Total hydrolysis was measured for 1 hr, and after correcting for non-enzymic hydrolysis, the results were expressed as per cent inhibition of control hydrolysis and plotted against the negative logarithm of the inhibitor concentration. The amount of inhibitor required to produce a 50 per cent inhibition in substrate hydrolysis was then determined, and designated as molar I_{50}. At least three different inhibitor concentrations were used in each experiment and duplicate flasks were included for each concentration. All compounds were run against the pseudocholinesterase–acetylcholine system; the other two substrate–enzyme systems were run only where desired for comparison purposes.

RESULTS AND DISCUSSION

The results obtained are summarized in Tables 1 and 2. These will be discussed briefly in terms of the influence of structural variables on activities.

Ample evidence exists in the published literature that among series of polymethylene bis-quaternary ammonium salts, there is a gradual rise in anticholinesterase activity with increase in length of the hydrocarbon chain separating the quaternary nitrogen atoms[7, 8]. Our data also show that among quite differently substituted sets of polymethylene bis-quaternaries, anticholinesterase activities generally increase with chain length within the limits tested (cf. I, II, III; VII, VIII, IX; XVI, XVII, XVIII; XX, XXI).

Although an increase in chain-length enhances anticholinesterase activities, it is equally evident from the data that among bis-quaternaries of equal hydrocarbon chain length, there is often a marked increase in anticholinesterase activities associated with an increase in lipophilic characteristics of the terminal substituents on the

* Winthrop Laboratories bovine erythrocyte acetylcholinesterase.
† Difco Laboratories horse serum.

TABLE 1. INFLUENCES OF VARIATIONS IN LIPOPHILIC CHAIN AND ONE CATIONIC HEAD IN $R^{\oplus}-(CH_2)_n-N^{\oplus}(CH_3)_3 \cdot 2\,Br$

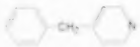
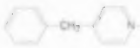
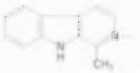
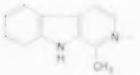
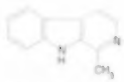
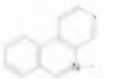
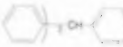
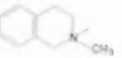
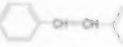
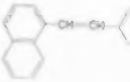
Compd.	$R^{\oplus}$	n	Anticholinesterase I_{50}			Mouse i.v. L.D. 50 (mg/kg)	Mouse i.v. E.D. 50‡ (mg/kg)
			BEChE AcCh*	Pseudo ChE† AcCh	Pseudo ChE† BuCh		
(I)	$(CH_3)_3N-$	3	—	$4{\cdot}5 \times 10^{-2}$	—	100	38
(II)	$(CH_3)_3N-$	6	1×10^{-2}	$6{\cdot}9 \times 10^{-3}$	—	22	16
(III)	$(CH_3)_3N-$	10	$5{\cdot}9 \times 10^{-4}$	$5{\cdot}1 \times 10^{-4}$	—	0·8	0·35
(IV)		3	—	$1{\cdot}1 \times 10^{-3}$	—	180	—
(V)		3	$> 10^{-3}$	$4{\cdot}9 \times 10^{-4}$	$> 10^{-3}$	40	27
(VI)		6	—	$1{\cdot}1 \times 10^{-4}$	—	7·5	3·4
(VII)		3	$5{\cdot}7 \times 10^{-4}$	$1{\cdot}3 \times 10^{-4}$	$2{\cdot}9 \times 10^{-4}$	27	24
(VIII)		6	$6{\cdot}0 \times 10^{-5}$	$5{\cdot}6 \times 10^{-5}$	$5{\cdot}2 \times 10^{-4}$	2·7	1·8

Table 1.—*continued*

Compd.	R ⊕	*n*	Anticholinesterase I_{50}			Mouse i.v. L.D. 50 (mg/kg.)	Mouse i.v. .D. 50‡ (mg/Kg)
			BEChE AcCH*	Pseudo ChE† AcCH	Pseudo ChE† BuCH		
(IX)		10	$1{\cdot}6 \times 10^{-5}$	$4{\cdot}7 \times 10^{-7}$	$6{\cdot}5 \times 10^{-6}$	2·3	0·8
(X)		3	—	$1{\cdot}3 \times 10^{-3}$	—	55	—
(XI)		3	—	$3{\cdot}8 \times 10^{-6}$	—	14	10
(XII)		3	—	$6{\cdot}6 \times 10^{-4}$	—	2·5	1·6
(XIII)		3	$5{\cdot}3 \times 10^{-3}$	$3{\cdot}2 \times 10^{-4}$	$2{\cdot}1 \times 10^{-4}$	2·3	1·2
(XIV)		3	—	$6{\cdot}5 \times 10^{-3}$	—	100	73
(XV)		3	—	$4{\cdot}0 \times 10^{-4}$	—	17	9·5
(XVa)		3	—	$2{\cdot}3 \times 10^{-5}$	—	1·2	0·88

* Bovine erythrocyte cholinesterase, acetylcholine substrate.
† Horse-serum cholinesterase, acetylcholine or butyrylcholine substrate.
‡ Intravenous dose causing 50 per cent of the mice to fall off inclined screen.

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TABLE 2. INFLUENCES OF VARIATIONS IN LIPOPHILIC CATIONIC HEADS AND CHAINS IN $R^{\oplus}-(CH_2)_n-R^{\oplus}$ .2 $Br^{\ominus}$

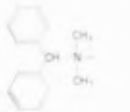
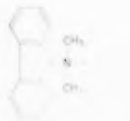
Compd.	$R^{\oplus}$	n	Anticholinesterase I_{50}			Mouse i.v. L.D. 50 (mg/kg)	Mouse i.v. E.D. 50 (mg/kg)‡
			BEChE AcCh*	Pseudo ChE†			
				AcCh	BuCh		
(XVI)		3	—	$5{\cdot}6 \times 10^{-4}$	—	39·5	19
(XVII)		6	—	$5{\cdot}6 \times 10^{-5}$	—	3·5	2·9
(XVIII)		10	—	$6{\cdot}5 \times 10^{-6}$	—	3·9	2
(XIX)		6	$5{\cdot}4 \times 10^{-5}$	$2{\cdot}1 \times 10^{-6}$	$1{\cdot}3 \times 10^{-6}$	1·5	—
(XX)		3	$4{\cdot}1 \times 10^{-4}$	$2{\cdot}4 \times 10^{-6}$	—	6·4	4·0

Table 2. *(continued)*

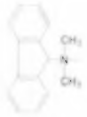
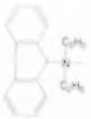
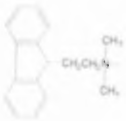
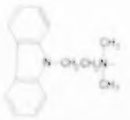
Compd.	R ⊕	*n*	Anticholinesterase I_{50}			Mouse i.v. L.D. 50 (mg/kg)	Mouse i.v. E.D. 50 (mg/kg)‡
			BEChE AcCH*	Pseudo ChE†			
				AcCh	BuCh		
(XXI)		6	1×10^{-5}	$3{\cdot}3 \times 10^{-7}$	$3{\cdot}2 \times 10^{-7}$	1·76	1·26
(XXII)		6	$2{\cdot}1 \times 10^{-4}$	$2{\cdot}9 \times 10^{-6}$	$3{\cdot}7 \times 10^{-6}$	30	—
(XXIII)		6	$6{\cdot}8 \times 10^{-6}$	$3{\cdot}0 \times 10^{-7}$	—	1·16	0·72
(XXIV)		6	1×10^{-5}	$1{\cdot}3 \times 10^{-7}$	$4{\cdot}1 \times 10^{-8}$	0·7	0·5
(XXV)		6	$5{\cdot}9 \times 10^{-5}$	$6{\cdot}7 \times 10^{-8}$	—	5·1	3·3

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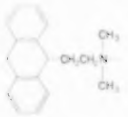
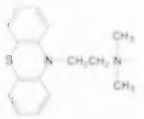
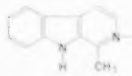
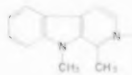
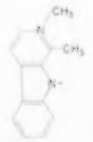
TABLE 2.—*continued*

Compd.	R ⊕	*n*	Anticholinesterase I_{50}			Mouse i.v. L.D. 50 (mg/kg)	Mouse i.v. E.D. 50 (mg/kg)‡
			BEChE AcCh*	Pseudo ChE†			
				AcCh	BuCh		
(XXVI)		6	$7{\cdot}3 \times 10^{-5}$	$6{\cdot}7 \times 10^{-5}$	—	1·5	1·2
(XXVII)		6	1×10^{-5}	$4{\cdot}0 \times 10^{-5}$	$5{\cdot}4 \times 10^{-5}$	7·3	6·5
(XXVIII)		6	—	$2{\cdot}0 \times 10^{-6}$	—	11·7	3·9
(XXIX)		6	—	$2{\cdot}4 \times 10^{-6}$	—	2·16	0·87
(XXX)		5	—	$4{\cdot}3 \times 10^{-7}$	—	1·19	0·75

* Bovine erythrocyte cholinesterase, acetycholine substrate.
† Horse-serum cholinesterase, acetylcholine or butyrylcholine substrate.
‡ Intravenous dose causing 50 per cent of the mice to fall off inclined screen.

"onium" nitrogen atoms (I, XVI, XX; II, XVII, XIX, XXI; III, XVIII; XV, XVa; VIII, XXVIII). Inasmuch as an increase in length of the hydrocarbon chain also increases the relative lipophilic:hydrophilic ratio in the total molecule, it becomes suspect that the influence of the chain-length is not exclusively, if at all, that often attributed to it of permitting the molecule to assume inter-cationic charge positioning more favourable for bonding of the molecule to complementary anionic receptor sites. It is evident from a comparison of (V), (XII) and (XIII) that an increase in total lipophilic bulk alone is not sufficient to influence anticholinesterase activity; however, among compounds with the same chain-length between nitrogen atoms, and with about the same mass of lipophilic terminal substitution, the configuration of the lipophilic mass appears to be quite important (XI vs. V, XII or XV). The influence of configuration of the lipophilic mass is also quite evident in the symmetric bis-quaternary series (XIX vs. XXI). It previously has been observed[2] that relatively planar, lipophilic substitution is more favourable for curarimimetic activity. At first glance it would appear that planarity is also important in contributing to anticholinesterase activity (XIX vs. XXI) but this would not explain the high activity of non-planar (XXVI) and (XXVII). A comparison of the activities of (II), (XVII), (XIX), (XXI), (XXIV), (XXV), (XXVI) and (XXVII) would support a contention that an increase in lipophilic substitution on the two "onium" nitrogen atoms increases anticholinesterase activities and that the increase is greater with rigid, suitably situated structures approaching planar and dome-shaped configurations. Anticholinesterase activity appears to be far less dependent upon planarity of lipophilic substitution than is curarimimetic action.

With the exception of (XXII), all of the compounds are quaternaries which under the conditions of the experiments are essentially completely ionized. The bis-tertiary amine (XXII) is the order of a tenth as active a cholinesterase inhibitor as its quaternary analogue (XXI). Among curarimimetics[9-12] and hypotensive agents[4] a high concentration of charge centered about the "onium" nitrogen atoms usually has a favourable influence on intensity of activity. With the present limited group of compounds, anticholinesterase activity actually is somewhat greater in the members of analogue pairs in which the cationic charge is less concentrated or more diffuse (IV vs. I, X vs. XIV). Among the three carboline derivatives, (XXVIII), (XXIX) and (XXX), toxicity or curare-like activity is higher with the latter two completely ionized structures[4], anticholinesterase activity is more influenced by the relationship of the onium heads to the linking chain.

Among acetylcholine receptors it might be expected that those situated at the myoneural junction (essentially an extracellular site) would resemble most closely in accessibility those situated on extracellular cholinesterases. The favourable influence of lipophilic characteristics on curarimimetic action has been discussed elsewhere[2, 13] and it has been pointed out that increases in length of the linking hydrocarbon chain in bis-quaternary substituted alkanes have a more favourable influence on intensity of activity than do corresponding lipophilic increments on the terminal onium heads[13]. From the tabulated data, it similarly may be concluded that increase in chain-length increases anticholinesterase activities more than might be expected from the relative influences of equivalent lipophilic increments on the terminal onium heads. However, the addition of nearly planar terminal substituents has a much greater favourable influence on both types of activity than does a similar increment in lipophilic non-

planar structures. Furthermore, among bis-quaternaries with appreciable terminal lipophilic substitution, a comparison of C_3, C_6 and C_{10} homologues shows a sharp increase in curarimimetic activity from C_3 to C_6 with little additional change at C_{10} whereas anticholinesterase activities show a pronounced increase at each level (VII, VIII, IX, XVI, XVII, XVIII). Among bis-quaternaries with less bulky terminal substituents, the C_3-C_6-C_{10} transition also shows a regular increase in anticholinesterase activity but the more marked increase in neuromuscular blocking action occurs in the C_6-C_{10} transition (I, II, III). From this it would appear that acetylcholine receptors at cholinesterase surfaces and at the myoneural junction have an affinity for bis-quaternaries which increases in a generally parallel fashion with increase in lipophilic characteristics of either or both the substituents on the terminal onium heads and the linking structure. The departure from parallelism which occurs with the C_6-C_{10} transition among the bulky, lipophilic substituted homologues may be a reflection of the influence of the two factors mentioned in the introduction, namely distribution and accessibility. Although the myoneural junction may be considered as an extracellular site, it probably is situated in a relatively confined location in comparison with the free accessibility of extracellular cholinesterases. The item of distribution becomes more significant in an *in vivo*-system and increased lipophilic bulk in the quaternaries would make them more susceptible to dilution by adsorption at lipophilic sites of loss.[13, 14] The proportionately more favourable influence of planarity on curarimimetic than on anticholinesterase activity also may be more related to greater steric hindrance to access of the less planar groups to a confined site than to differences in affinity for the acetylcholine receptors.

Some of the unsymmetric bis-quaternaries included in this study represent rather potent hypotensive agents which may have both a central and a peripheral ganglionic component of action.[1, 4, 5] In either event, action at such acetylcholine receptors would presume that these hypotensive agents to some extent pass through biological membranes. Unsymmetric bis-quaternaries of type $B^{\oplus}$-$CH_2CH_2CH_2N^{\oplus}(CH_3)_3$ show hypotensive activity with $B^{\oplus}$ as a lipophilic onium head of between certain minimum and maximum limits of size. Only as one approaches or exceeds this maximum order of size of $B^{\oplus}$ do anticholinesterase and curarimimetic activities become significant. Here again the inherent affinity for the acetylcholine receptors might generally be increased with increased lipophilic bulk of $B^{\oplus}$ but with the ganglion or central sites enveloped by membrane sheaths, distribution is complicated by much more limited accessibility than at the myoneural junction and correlation between anticholinesterase and hypotensive activities would occur in an even more limited range of structural parellelisms. The order of hypotensive activity of these unsymmetric bis-quaternaries is approximately (XIII), (XIV), (XVa), (XII), (XI), (VII), (II, V, X), (XV), (VIII), (I, IV).*

With the compounds of this group and under the test conditions used, the pseudocholinesterase preparation was in general more sensitive to inhibition than was the acetylcholinesterase. We hesitate to draw any correlation between structure and specific anticholinesterase activities beyond this at present. It is noted that I_{50} values of the order of magnitude of 10^{-8} represent an approximate peak in activity. This may be a reflection of the lowest concentration of maximally effective reversible

* For details pertaining to hypotensive and ganglionic blocking activities, reference should be made to the cited bibliography.

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inhibitor required to block enough enzymatic receptors to inhibit enzyme activity by 50 per cent. The high activity of compounds containing relatively rigid, nearly planar dome-shaped, non-polar substituents may result from an increase in van der Waals bonding energies and from a shielding effect provided by such structures whereby the adsorbed molecule is protected from displacing ions and the complementary enzyme surface is screened from the substrate. This shielding effect would be expected to be provided best by rigid structures of appreciable size but yet not so large that the compound is associated in solution or that it cannot approach the enzyme bonding sites close enough for effective bonding.

During its evaluation in humans as a curarimimetic, it was found that one of these compounds (XXI) (hexafluorenium bromide or Mylaxen®), produced a marked potentiation of the action of succinylcholine. Techniques for applying this in practice have been reported by Arrowood and Kaplan[15] and Rizzi and Galeotto[16]. Foldes *et al.*[17, 18] have measured the anticholinesterase activity of (XXI) and have developed a system for its use as a succinylcholine potentiator based on quantitative studies of time–action measurements of the inhibitory effects of (XXI) on human serum cholinesterases. Other ester quaternaries such as caproylcholine[19] and suxethonium[18] are potentiated in a similar manner. The otherwise ultra-short acting suxethonium can be extended in action by (XXI) so that it can be successfully used for the production of prolonged muscle relaxation.[18] The absence of stimulating or blocking effects of (XXI) on acetylcholine receptors other than those of serum cholinesterases provides a potentiation which is remarkably free of untoward reactions.

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STRUCTURE MOLÉCULAIRE ET ACTIVITÉ OESTROGÈNE

I. GRUNDLAND

Varsovie Pologne

(*Received* 31 *March* 1959)

Abstract—On analysis of the physiological process of rutting two important factors which accompany oestrous become apparent: (a) a capacity for developing hyperplasia which is induced or already exists in the organism; (b) a condition, brought about by a specific molecular configuration of the active substance, which is in equilibrium with the fat fraction and with the polar groups attached to the active molecule. This equilibrium favours the transport of lipids and consequently favours hyperplasia particularly at the level of the active organ. Investigation shows that different oestrogenic substances, both physiological and synthetic, reproduce these same conditions in the organism.

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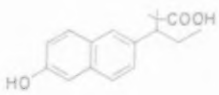
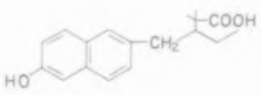
DEPUIS des années, on assiste à l'éclosion de nombreuses substances douées d'un pouvoir oestrogène. Alors que l'activité oestrale était auparavant l'apanage des composés à noyau pentano-phénantrène, les dérivés de l'acénaphtène,[1] de l'anthracène,[2] des stilbènes,[3] du triphényl-éthylène,[4] du naphtalène[5, 6] se sont successivement révélés oestrogènes et, parfois même, à un degré supérieur à la folliculine. Devant la dissemblance structurale de ces substances, il est apparu difficile de rattacher leurs propriétés physiologiques à une configuration chimique déterminée.



Composé actif



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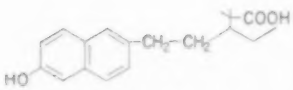

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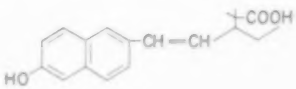

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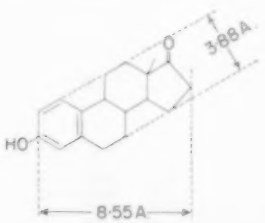
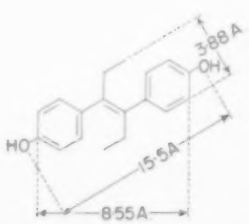




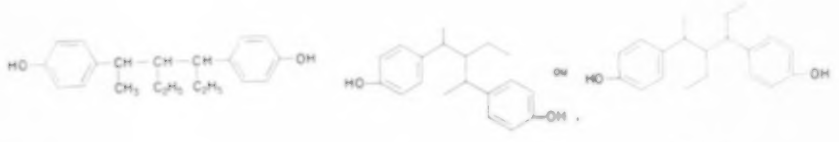
La ressemblance des radiogrammes (et la juxtaposition des formules stéréochimiques) de l'oestradiol d'une part et, d'autre part, de l'hexoestrol,[8] du stilboestrol (éther diméthylique),[9] ou de certaines coumarines,[10] s'est avérée, malgré l'intérêt évident qu'elle présente, incapable d'englober les autres familles des corps mentionnés. Elle a même montré quelques irrégularités quant aux prévisions qu'elle semblait autoriser. Ainsi l'éthyl *p*-hydroxyphényl-2-hydroxy-6-naphtalène et son dérivé tétrahydrogéné en 1-2-3-4 sont très peu actifs (activité = 1/10.000 de celle de l'hexoestrol) alors que le dérivé méthylé est déjà actif chez la souris à la dose de 10 μg.

Pour Schueler,[7] la propriété oestrogène serait liée à la présence de deux hydrogènes actifs (par exemple phénoliques) distants d'environ 8,55 Å dans une grosse molécule sans spécificité (l'oestradiol, le hexestrol, l'acide α:α-diméthy-β-éthyl allénolique, etc. . . .). Ainsi à titre d'exemple l'activité oestrogène dans la série allénolique semble dans un certain rapport être liée au fait remarqué d'une distance fixe-utile de 8,55 Å entre les groupements polaires—puisque des variations dimensionnelles tendant à modifier cette distance réduisent ou suppriment l'activité physiologique.[18]

VARIATIONS DE DISTANCE ENTRE GROUPEMENTS POLAIRES DANS LA SÉRIE ALLÉNOLIQUE ET POUVOIR OESTROGÈNE

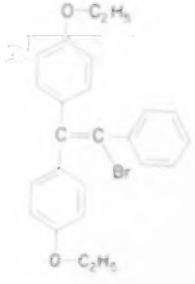
L'analogie de la forme spatiale moléculaire de nombreuses substances synthétiques avec l'hormone folliculaire a été soulevée. Cette remarque souffre des exceptions (e.g. l'acide α:α-diméthyl-β-propyl-allénolique présente une activité accrue de 50% par rapport à son homologue avec un radical éthyl en β alors qu'il s'écarte bien plus du schéma structural de l'hormone naturelle,[19]) et même admise, laisse entier le problème du mécanisme d'action.*

* Le benzoestrol qui s'écarte de la structure de l'hormone naturelle a cependant une activité oestrogène intermédiaire se plaçant entre celle de l'oestrone et de l'oetradiol. On peut à la rigeur le figurer de manière à ébaucher des cycles potentiels et à ressortir une certaine analogie spatiale—ou la distance entre les deux groupements polaires phénoliques approche celle des hormones.

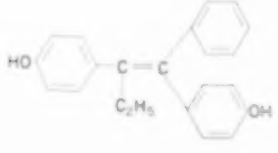


Forme *a* Forme *b*

Rien de semblable n'est cependant pas possible avec le triphénylchloréthylène qui pourtant est assez actif.

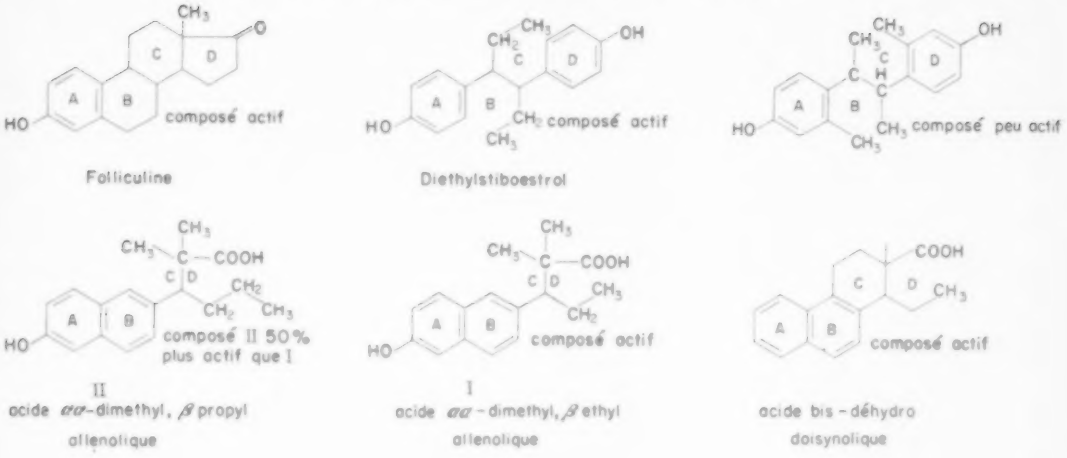


actif à 20μg



actif à 5μg

ANALOGIE SPATIALE (CYCLES POTENTIELS)



Il est apparu, par suite, nécessaire, afin de saisir le mécanisme d'action des substances à pouvoir oestrogène, de rechercher des conditions très générales qui s'attachent à ces molécules lors des modifications provoquées dans l'organisme récepteur.

Ces modifications de l'organisme récepteur au moment de l'oestrus impliquent grosso modo, lors de cette manifestation physiologique importante, d'une part, un retentissement sur l'ensemble de l'organisme, d'autre part, des modifications locales caractéristiques.

En ce qui concerne les modifications locales caractéristiques, on est frappé par le fait qu'au moment de l'oestrus (chez la Rate blanche), il existe une véritable migration des lipides à travers la muqueuse de l'utérus et du vagin, ceci en fonction de leur activité.[11,*]

Ainsi la corne utérine et le vagin participent activement à la sécrétion oestrale, cependant avec des différences d'aspect déterminées par leur structure. Pendant

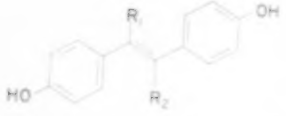
* A noter que la date d'apparition de la puberté (jugée d'après la date d'ouverture du conduit vaginal chez la rate) est fortement influencée par la carence du régime en graisses. Voici un tableau cité d'après Deuel et Greenberg in *Fortschr. Chem. org. Naturstoffe* **6**, 45 (1950).

	Moyenne de jours en lesquels la maturité est acquise à dater de la satauration ad libitum avec un régime contenant : graisses				
	0%	5%	10%	20%	40%
Rates normales, âgées de 3 semaines au départ de l'expérience, soumises au régime ad libitum à dater du sevrage	60,2	49,0	45,5	44,8	44,1
Rates carencées, âgées de 15 semaines au départ (après 12 semaines de régime carencé)	14,7	11,9	10,5	7,7	9,8

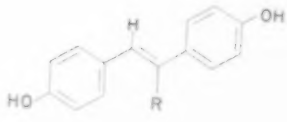
l'oestrus, les cornes se gonflent d'un liquide muqueux acellulaire, dans lequel on peut parfois reconnaître des leucocytes. Le frottis exécuté avec le produit de sécrétion muqueux de la corne, fixé et coloré au Flemming, montre l'existence de gouttelettes de graisse libres. Ces gouttelettes apparaissent dans les corne dès le pro-oestrus et s'y trouvent en nombre d'autant plus important que l'animal est à un stade de rut plus caractérisé. Elles proviennent de la migration de lipides décelables à l'état de gouttelettes, tant basales qu'apicales, dans le revêtement épithélial cylindrique des cornes.

Au niveau du vagin, lors de la desquamation des couches superficielles, ce sont des placards cornés qui caractérisent la sécrétion oestrale. On retrouve les lipides dans ces éléments cellulaires sous forme de fine gouttelettes soudanophiles (coloration au noir Soudane après fixation au Flemming). D'ailleurs, l'épithélium stratifié de la portion commune de l'utérus et du vagin se colore uniformément en grisâtre par le noir Soudane sur les pièces fixées par la méthode de Ciaccio. La couche superficielle de l'épithélium stratifié se colore plus intensément quand elle se kératinise. Ainsi, il y a, au moment de l'oestrus, élimination de graisses dans les éléments cellulaires desquamés et le produit de sécrétion muqueux des cornes. Cela incite à penser qu'il

TABLEAU 1. ACTIVITÉ OESTROGÈNE EN FONCTION DES VARIATIONS DE LA FRACTION PARAFFINIQUE DES MOLÉCULES ACTIVES[25]



R_1	R_2	Dose (mg)	de Réponses positives (%)	Unités rat par g
H	H	5	80	
		10	100	140
CH_3	CH_3	0,02	80	
		0,03	100	40.000
CH_3	C_2H_5	0,0005	30	
		0,001	100	1.000.000
C_2H_5	C_2H_5	0,0003	80	
		0,0004	100	3.000.000
CH_3	nC_3H_7	0,0005	80	
		0,001	100	1.000.000
C_2H_5	nC_3H_7	0,001	trace	
		0,01	100	300.000
nC_3H_7	nC_3H_7	0,01	75	
		0,1	100	50.000
Propényle	Propényle	0,01	30	
		0,1	100	20.000
nC_4H_9	nC_4H_9	0,01	néant	
		0,1	40	5.000
C_2H_5	Cétyle	1	80	
		3	100	500



R	Dose (mg)	de Réponses positives (%)	Unités rat par g
H	10	100	140
CH_3	1	100	1.000
C_2H_5	0,1	50	5.000
nC_3H_7	0,1	néant	
	0,2	100	5.000
$isoC_3H_7$	0,2	néant	
	0,5	100	2.000
nC_4H_9	0,5	20	
	1	100	1.000
$isoC_4H_9$	0,2	40	
	0,5	100	2.000
nC_5H_{11}	5	80	
	10	100	100

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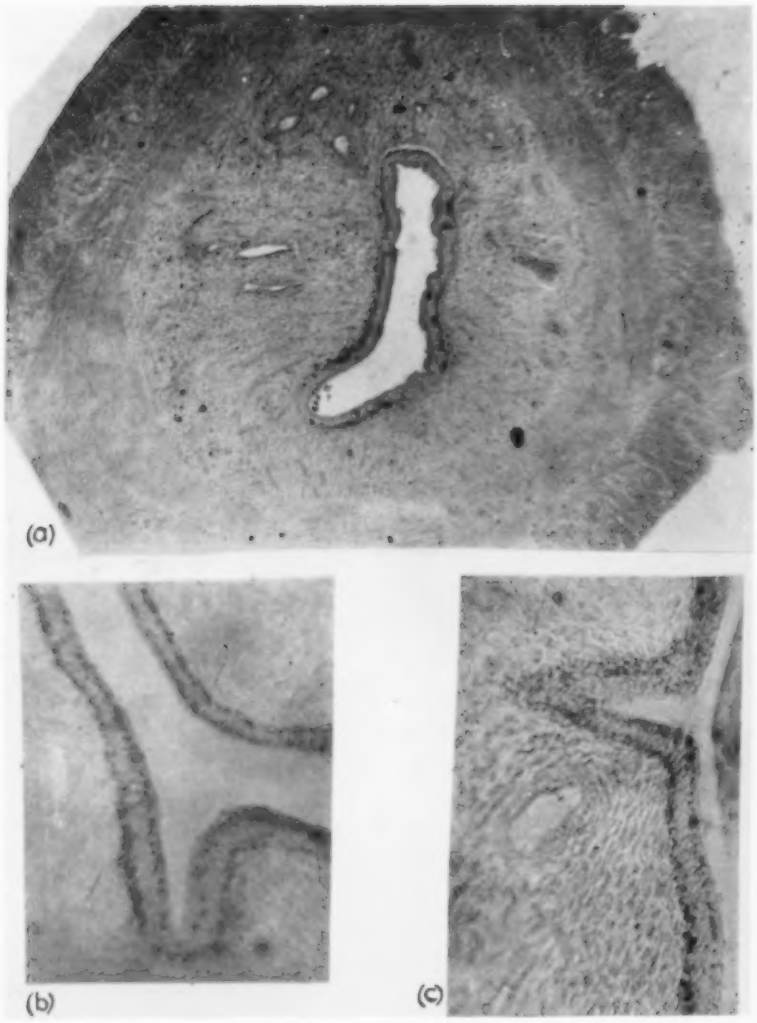

FIG. 1. (a) Corne utérine, (b) pro-oestrus (fin), (c) gouttelettes basales et apicales dans l'epithelium. Fixation Ciaccio, coloration noir Soudane.

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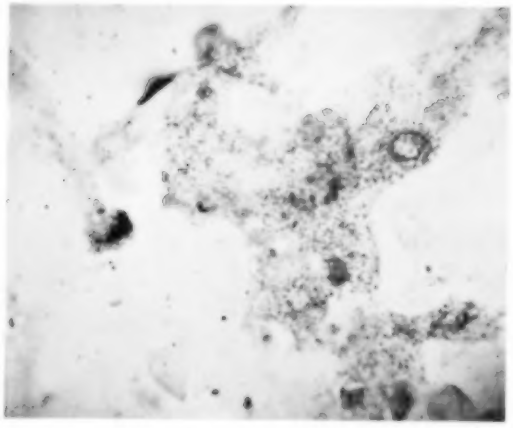

FIG. 2. Frottis pendant l'oestrus. Gouttelettes de graisse intra-cellulaire. Fixation Flemming, coloration noir Soudane.

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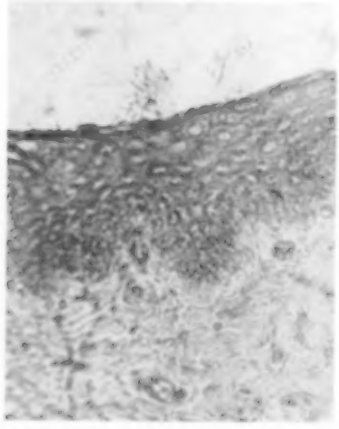
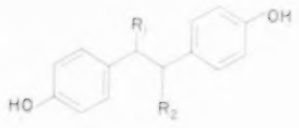
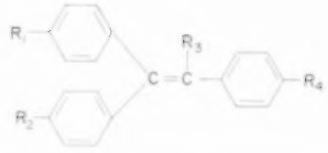


FIG. 3. Proestrus (fin) Mugueuse Vaginale Ciaccio, coloration noir Soudane.

TABLEAU 1—*cont.*



R_1	R_2	Dose (mg)	de Réponses positives (%)	Unités rat par g
H	H	100	—	—
CH_3	CH_3	0.0002	néant	—
		0,0005	100	2.000.000
C_2H_5	C_2H_5	0.0002	100	5.000.000
C_3H_7	C_3H_7	0.001	néant	
		0,005	100	200.000
H	Cétyle	100	100	10



R_1	R_2	R_3	R_4	Activité oestrogène
H	H	H	H	1.000 μg (souris)
H	H	CC	H	500 μg (souris)
H	H	C_2H_5	H	2.500 U.R. g
OH	H	C_2H_5	OH	400.000 U.R. g
OH	H	H	OH	10.000 U.R. g
OH	H	H	H	4.000 U.R. g
OH	H	C_2H_5	H	30.000 U.R. g
OCH_3	OCH_3	Br	H	20 μg (souris)
OC_2H_5	OC_2H_5	Br	H	20 μg (souris)
H	H	CN	H	1.000 μg (par 20 g de rat)
H	H	CH_3	H	20 μg (par 20 g de rat)
OCH_3	OCH_3	Br	OCH_3	20 μg (souris)
OCH_3	OCH_3	CC	OCH_3	1.000 μg (rat)
OCH_3	H	H	OCH_3	200 μg (souris 25% réponses)
OCH_3	OCH_3	CH_3	OCH_3	10 μg (souris 45% réponses)
OCH_3	OCH_3	C_2H_5	OCH_3	40 μg (souris 90% réponses)

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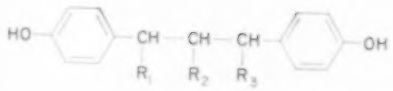
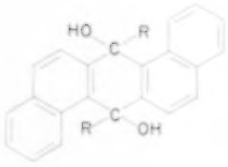
TABLEAU 1—*cont.*



R_1	R_2	R_3	Isomère	Point de fusion (°C)	Activité oestrogène (mg)
H	H	H			100
C_2H_5	CH_3	CH_3	B-2	141–143	0,50
C_2H_5	CH_3	C_2H_5	A	résine	1,0
			B-2	126–127	5,0
C_2H_5	C_2H_5	C_2H_7	A-2	102	2,0
			B-2	143–144	2,0
C_2H_5	C_3H_7	C_2H_5	A	résine	0,60
			B	résine	1,0
C_2H_5	C_2H_5	C_2H_5	A-2	144	1,0
			B-2	138–139	0,50
			B-2	154–155	0,10
C_3H_7	C_2H_5	CH_3	A-2	132	0,40
			B-2	155	0,013
C_2H_5	C_3H_7	CH_3	A-2	68–72	0,10
			B-2	121–122	0,005
C_2H_5	C_2H_5	CH_3	A-1	résine	0,010
			A-2	75	0,005
			B-1	144	0,035
			B-2	162	0,0008

TABLEAU 2. [23, 24]

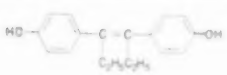
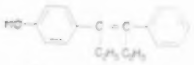
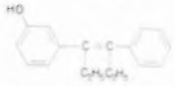
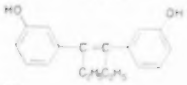
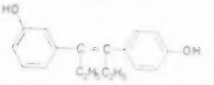
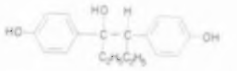
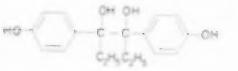
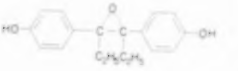
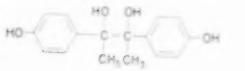
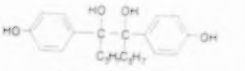
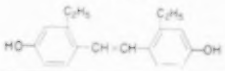


R	dose (mg)	Réponses positives (rat) (%)
CH_3	100	néant
C_2H_5	1	100
n-C_3H_2	1	100
	0,05	50
n-C_4H_3	10	90
	1	10
n-C_6-H_{10}	100	néant
n-C_6-H_{13}	100	néant

existe un rapport entre le caractère liposoluble commun aux substances oestrogènes et l'élimination des graisses au moment du rut. Les oestrogènes comportent en effet dans leur molécule une fraction paraffinique d'une part et des groupements polaires d'autre part. A leur fraction paraffinique s'attacheraient les graisses et les groupements polaires acquis serviraient à maintenir ce complexe en suspension dans le sang. Arrivée au niveau du tractus génital, cette émulsion se romprait, au contact et en faveur des enclaves lipidiques cytologiques, libérant les lipides que nous voyons dans les microphotographies suivre la vie fonctionnelle de l'organe. L'oestrogène, repris par le courant circulatoire, irait amener d'autres molécules lipidiques vers le tractus génital,

TABLEAU 3

VARIATIONS DE LA FRACTION POLAIRE DANS LA SÉRIE DE DÉRIVÉS DU STILBÈNE ET POUVOIR OESTROGÈNE

Substance active	Dose minimum efficace (rat) (μg)
	0,4
	1000
	9000
	9000
	4500
	50
	100
	1
	100.000
	10.000
	1000

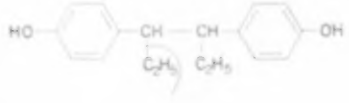
favorisant son fonctionnement. Le fait de retrouver d'une manière constante des graisses aux différents étages du tractus génital, et en fonction de son activité, prouve suffisamment la sensibilité de cet organe aux molécules lipidiques, et divers auteurs[12,*] ont déjà noté cette existence d'un pouvoir lipopexique de la muqueuse vaginale.†

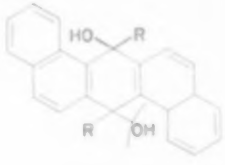
* Voir aussi: H. B. Van Dyke et G. Chen, *Amer. J. Anat.* **66**, 411 (1940). I. Rossman, *Amer. J. Anat.* **67**, 187 (1941).

† Certains auteurs ont montré d'autre part une certaine correlation, mais irregulière entre le pouvoir oestrogène et la lipémie.[27]

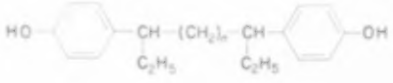
Cependant, il semble que, pour qu'une substance puisse intervenir activement afin de déclencher l'oestrus par ce processus, un certain équilibre est requis dans la structure moléculaire utilisée, équilibre entre la fraction paraffinique et les groupements polaires qui s'y attachent.

Ainsi, en parcourant les maintes substances oestrogènes sous l'angle de ce rapport entre la fraction paraffinique et les groupements polaires, on s'aperçoit que l'activité oestrogène de ces corps se trouve affectée par une transgression dans un sens ou autre (favorisant tantôt l'aspect polaire ou paraffinique de la substance examinée). A ne choisir qu'aux antipodes, on peut citer les exemples suivants:





Dans le cas du diéthyl-stilboestrol, la suppression d'un groupement éthyl allégeant la fraction paraffinique et accentuant encore l'aspect polaire de cette substance diminue considérablement le pouvoir oestrogène de ce corps.[13] Dans le cas du dibenzanthracène-diol (9:10-dihydroxy, 9-10-dialcoyl, 9:10-dihydro, 1:2-, 5:6:dibenzanthracène) par contre,[14] la suppression d'un hydroxyle diminuant l'aspect polaire et accentuant le caractère paraffinique de la molécule—déjà très marqué—fait disparaître le pouvoir oestrogène que la substance possédait au départ. Ceci en ce qui concerne les exemples pris aux antipodes. Mais cette remarque reste valable aussi pour des corps où l'équilibre entre la fraction paraffinique et les groupements polaires n'est pas aussi déformé. Ainsi pour le diéthyl-stilboestrol, l'introduction d'une chaîne polyméthylique[15] entre les deux noyaux (accentuant cette fois non pas l'aspect polaire comme ci-dessus, mais paraffinique de la substance) réduit considérablement le pouvoir oestrogène:



En ce qui concerne les groupements polaires il est a noter que par exemple dans la série doisynolique on allénolique le remplaçement de la fonction acide pour une fonction alcool primaire ou aldéhyde ne diminue que très peu l'activité oestrogène.[26] Dodds *et al.*[20] ont d'autre part montré que durant son intervention physiologique la molécule oestrogène active (dans l'occurrence l'hexoestrol tritié) intervient sous sa forme inchangée et que moins de 2% de la substance active subit dans l'organisme une oxydation.

Dans l'ensemble, il ressort de ces faits qu'un certain équilibre doit figurer entre la fraction paraffinique et les groupements polaires à l'intérieur d'une molécule afin que celle-ci garde le pouvoir oestrogène. C'est peut-être seulement sous cette condition que la substance oestrogène peut intervenir dans le mécanisme du transport des

lipides consommés lors du processus cellulaire de l'oestrus (tel qu'il a été observé dans l'étude cytochimique de l'organe effecteur). Etant par trop paraffinique, la substance oestrogène n'est pas à même de diffuser un autre lipide à travers la phase aqueuse (des humeurs) et ne peut servir à son transport; étant par trop polaire, elle ne s'attache pas par un lien assez solide à un lipide, se perd dans la phase aqueuse, et cette fois encore ne peut servir au transport des lipides. Il est intéressant à noter que le hexadéne, les dérivés de triphénylethyléne se fixent sur les graisses de l'animal où ils forment des reserves ce qui expliquerait leur pouvoir oestrogène prolongé; le diéthyl-stilboestrol et le hexoestrol ne forment pas de pareilles reserves aussi ils ne donnent pas lieu à des effets oestrogènes prolongés[22].

Une preuve de l'attraction entre lipide et substance oestrogène formant un complexe instable (par forces de Van der Waals) a pû être donnée lors de l'étude des films mono-moléculaires mixtes de l'hexestrol.[16]

Ainsi, alors qu'un film monomoléculaire de propionate d'hexestrol peut être suivi jusqu'à une pression de 3,5 dyn/cm et présente alors une surface de 100 Å²/mole, au-delà de cette pression le film gazeux se collabe, d'où impossibilité de continuer les mesures. Cependant, lorsqu'on projette sur le plan de l'eau un mélange contenant deux molécules d'acide oléique pour une molécule de propionate d'hexestrol—pour des raisons de commodité de dessin la courbe tracée représente la somme d'un acide oléique + ½ propionate d'hexestrol—on obtient un film superficiel résistant jusqu'à 7 dyn, avec lorsqu'on accentue la pression rupture du complexe, expulsion vers la phase aqueuse de propionate d'hexestrol et subsistance sur le plan de l'eau de l'acide oléique seul. L'augmentation de la résistance du film à la compression allant de

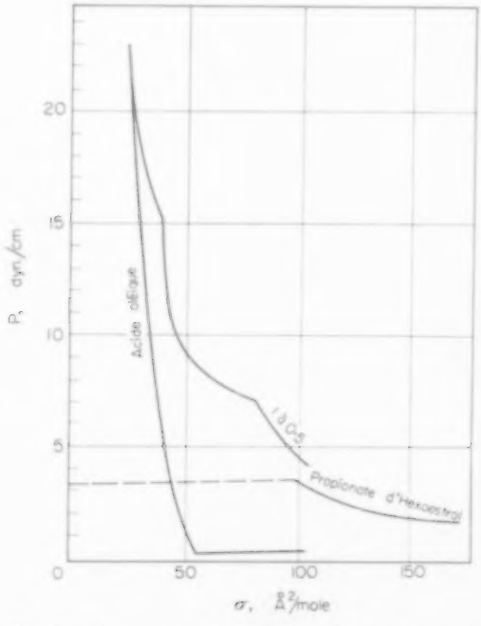


FIG. 4. Isotherme $P = f(\sigma)$ des films monomoléculaires mixtes acide oléique, propionate d'hexoestrol. Propionate d'hexoestrol, élement variable. Lectures des points toutes les 3 min (5 min après étalement) dans le sens de la compression.

3 dyn/cm jusqu'à 7 dyn/cm prouve une association instable entre l'hormone oestrogène de synthèse et somme toute une substance à caractère principalement paraffinique (un lipide). Le complexe ainsi formé serait susceptible de favoriser la diffusion et l'arrivée vers le tractus génital des lipides utilisés lors de l'oestrus. Ceci par suite de l'acquisition par les lipides à titre temporaire dans le complexe des groupements polaires de l'hormone.

Le complexe étant instable, cèderait les lipides aux enclaves lipidiques protoplasmiques existant déjà dans le tractus génital (l'adhésion latérale genre van der Waals étant plus forte dans le cas lipide-lipide que lipide-substance oestrogène) et l'hormone oestrogène libérée serait susceptible de resservir par répétition reitérée de ce même processus, servant comme intermédiaire et n'étant pas consommée elle même dans le cours du phénomène physiologique. On comprendrait alors la raison de son activité à des taux infinitésimaux (0,1 μg) alors que la manifestation physiologique provoquée est aussi considérable (et, de fait, il n'a pas été possible de révéler dans les lipides du tractus génital, par examen spectrographique, la présence des oestrogènes artificiels provoquant l'oestrus dans le cas par exemple du benzopyrène ayant pourtant un spectre très caractéristique).

Ainsi les diverses substances oestrogènes s'imbriquent dans le jeu physiologique de l'oestrus facilitant, du fait de leur structure moléculaire particulière la migration des graisses.

Cependant, lors de l'oestrus, il ya a également croissance cellulair puisqu'il y a hyperplasie des cornes utérines et formation des strates multiples dans l'épithélium vaginal qui desquame. Certes, les lipides se retrouvent partout dans les formations

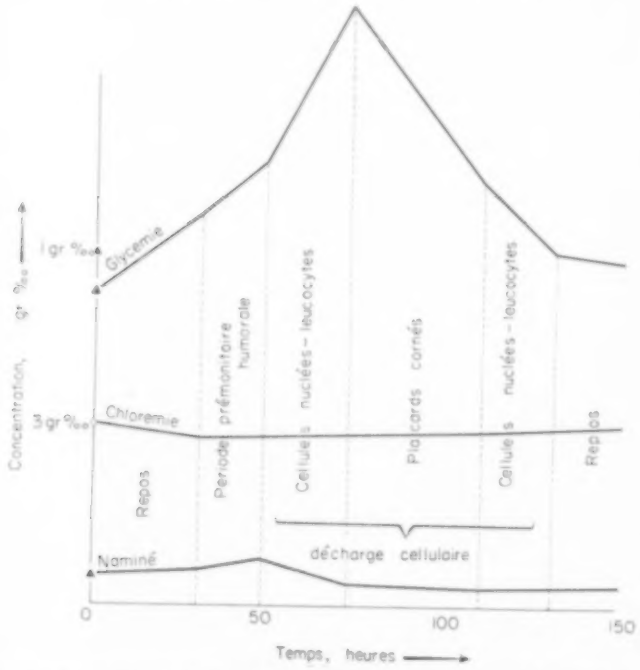


FIG. 5. Evolution en fonction du temps (en heuves) de l'oestrus aspect humoral et cytologique.

TABLEAU 4

Substance active		0 hr	48hr pro-oestrus	72 hr oestrus	110 hr	150 hr
Folliculine 1 mg	Glycémie (‰)	0,74	0,95	1,45		0,76
	Cl sang total (‰)	3,04	2,80	2,86		2,65
	Cl plasmatique (‰)	3,60	3,76	3,71		3,91
	N aminé par cm^3 (mg)	0,076	0,124	0,066		0,074
Rates non castrées	Eléments cellulaires du frottis	—	cellules nucléées	placards cornés	leucocytes	cellules nucléées
Dipropionate de diéthyldioxy-stilbène 2,5 mg	Glycémie (‰)	0,88	1,74	1,39		1,45
	Cl sang total (‰)	3,04	2,95	2,92		3,08
	Cl plasmatique (‰)	3,60	4,07	3,83		3,08
	N aminé par cm^3 (mg)	0,076	0,129			
Rates non castrées	Eléments cellulaires du frottis	—	placards cornés	placards cornés	placards leucocytes	placards cornés
3–4 Benzopyrène 100 mg (dans l'huile de sésame)	Glycémie (‰)	0,88	0,92	1,06	1,16	0,92
	Cl sang total (‰)	2,60	2,15	—	—	2,77
	Cl plasmatique (‰)	3,60	4,41	3,40	—	3,53
	N aminé par cm^3 (mg)	0,09	0,126			0,089
Rates castrées	Eléments cellulaires du frottis	—	cellules nucléées	placards cornés	cellules nucléées	cellules nucléées (peu)
Cycle oestral physiologique	Glycémie (‰)	0,88	1,26	1,70	1,20	0,88
	Cl sang total (‰)	3,04	2,83	2,94	—	3,04
	Cl plasmatique (‰)	3,59	3,95	3,90		3,60
	N aminé par cm^3 (mg)	0,076	0,116	0,077		0,076
Rates non castrées	Eléments cellulaires du frottis	—	cellules nucléées	placards cornés	cellules nucléées & anucléées (peu) leucocytes	

cellulaires nouvelles, mais les lipides seuls sont incapables d'expliquer et contribuer à la néoformation cellulaire.

Le jeu physiologique de l'oestrus comporte nécessairement une répercussion sur l'ensemble de l'organisme tendant à mobiliser les possibilités de néoformation cellulaire. Effectuant d'une part des mesures d'azote aminé, de glucose, de chlorures dans le sang total et au même moment effectuant des examens cytologiques des frottis de sécrétion vaginale chez des animaux durant le cycle oestral physiologique ou provoqué par des substances oestrogènes de synthése, il a été possible d'observer une hyperpolypeptidémie, hyperglycémie et un faible mouvement des chlorures en sens inverse.[17]

Dans le cas du rut normal ou provoqué, l'acmé du syndrome de perturbation chimique ainsi individualisé précède l'apparition des cellules desquamées sur les frottis respectifs, faisant penser que la mise en oeuvre du processus physiologique de décharge cellulaire sert à ramener vers la normale l'équilibre perturbé des composants du sang.

Cette fois, la perturbation de l'équilibre humoral (avec apparition de l'hyperpolypeptidémie) est parfaitement susceptible d'être le motif de la néoformation cellulaire qui, dans le cas particulier, apparaît dans le tractus génital et y est particulièrement favorisée par le flux des lipides qui y sont consommés—flux entretenu par

l'aptitude au transport des lipides résultant d'un équilibre particulier dans la structure moléculaire que possèdent les diverses substances à pouvoir oestrogène, réalisant par ailleurs le syndrome humoral également.

Ainsi, l'analyse du processus physiologique du rut fait apparaître les deux conditions majeures accompagnant l'oestrus: (a) l'aptitude à l'hyperplasie, (b) condition issue d'une structure moléculaire particulière favorisant l'hyperplasie au niveau de l'organe effecteur,—l'examen montre que les diverses substances oestrogènes tant physiologiques qu'artificielles (de synthèse) reproduisent dans l'organisme ces mêmes conditions.*

* Sur le plan de la chimie de synthèse la solution offerte par Dodds est parfaite et élegante dans la forme moléculaire et il n'existe actuellement aucun besoin réel à l'améliorer.

RÉSUMÉ

L'analyse du processus physiologique du rut fait apparaître deux conditions majeures accompagnant l'oestrus: (a) l'aptitude à l'hyperplasie crée ou existante dans l'organisme; (b) condition résultant d'une structure moléculaire particulière de la substance active consistant en un équilibre dans la structure moléculaire utilisée entre la fraction paraffinique et les groupements polaires qui s'y attachent, équilibre favorable au transport des lipides et par ce fait favorisant l'hyperplasie particulièrement au niveau de l'organe effecteur. L'examen montre que les diverses substances oestrogénes tant physiologiques qu'artificielles (de synthése) reproduisent dans l'organisme ces mêmes conditions.

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INFLUENCE OF 6-AZAURACIL ON THE METABOLISM OF *BACILLUS CEREUS*

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Abstract—The metabolic effects produced by the treatment of *Bacillus cereus* with azauracil have been investigated. The treated bacteria were observed to grow at a lower rate and to synthesize DNA, RNA protein and cell wall material in smaller amounts than in the untreated control culture. The studies suggest that the site of inhibition of this pyrimidine analogue is the mechanism which is involved in the conversion of orotic acid to uracil. Thymidine (but not thymine) in addition to uracil, uridine, uridylic acid, cytosine, cytidine and cytidylic acid reduces the inhibition resulting from the treatment with azauracil. The results suggest that in *B. cereus* thymidine may be converted to uracil or a uracil derivative. Azauracil does not seem to exert a specific effect upon the formation of cell wall material in *B. cereus*.

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1. INTRODUCTION

SEVERAL recent publications have reported the ability of 6-azauracil to inhibit the growth of certain micro-organisms[1, 2] and various experimental neoplasms[3] in mice. In this study an attempt has been made to investigate the metabolic effects produced by the presence of this pyrimidine analogue in a culture of *B. cereus*.

In view of the studies of Park[4], in which he demonstrated that the addition of penicillin to a culture of *Staphylococcus aureus* resulted in the production of bacteria lacking cell wall and an accumulation of uridine-containing nucleotides, the hypothesis has become prevalent that uridine nucleotides are intimately related to cell wall synthesis. This argument has become all the more convincing since the discovery that the nucleotides which accumulate contain compounds[5, 6, 7] which are found normally in cell wall material. With the above findings in mind, a study of cell wall formation was made in the presence of the pyrimidine analogue 6-azauracil.

2. MATERIALS AND METHODS

Bacillus cereus, strain NCTC 569, was used in all the experiments. Cultures for experimental work were grown in the medium described by Pollock *et al*[8]. Roux bottles containing 50 ml of medium were inoculated and incubated at 30 °C for 17–19 hr. The bacteria were harvested by centrifugation and resuspended in fresh medium. The concentration of bacteria at the beginning of an experiment was such that the optical density was approximately 0·350. Growth was determined by turbidimetric measurements in a Hilger Spekker absorptiometer.

Analytical methods

Hexosamine was determined by a modification of the method of Elson and Morgan

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proposed by Blix[9]. Protein measurements were performed using the method of Lowry *et al*[10]. Desoxyribonucleic acid was measured using the procedure of Ceriotti[11] and ribonucleic acid by determining the colour produced with orcinol.[12]

Samples taken for analysis were added to an equal volume of cold 10% TCA. Extraction of acid soluble material and washing of the precipitated material with 5% TCA was carried out in an ice bath. In experiments in which incorporation of a radiocative compound was followed, the TCA used for precipitation and for extraction always contained a low concentration of the unlabelled material.

Labelled compounds. Orotic acid 2-C-14 and uracil 2-C-14 were obtained from the Radiochemical Center, Amersham, England. The incorporation of these labelled compounds into acid-insoluble material was measured. The bacteria were precipitated with an equal volume of 10% TCA (containing the unlabelled precursor) and then filtered through Millipore filters and washed several times with 5% TCA.

Pyrimidine analogues. The following pyrimidine compounds were tested for their effect on the growth of *B. cereus*: 6-azauracil (obtained from California Research Foundation), 2-thiouracil (Schwartz Laboratories, New York), isobarbituric acid (Sigma Chemicals, St. Louis), nitro-orotic acid (Sigma Chemicals, St. Louis), 5-fluorouracil and 5-fluo-roorotic acid (Hoffman-La Roche), and 5-bromouracil (Light & Company). Thymidine (Calif. Foundation for Biochem. Research), cytosine (Schwartz Laboratories), cytidine (Schwartz Laboratories), uracil (Hoffman-La Roche), uridylic acid and uridine (Schwartz Laboratories) were examined for their ability to reverse the inhibition produced by 6-azauracil.

3. RESULTS

Initially various pyrimidine analogues were tested for their influence upon growth of *B. cereus*. Table 1 lists the compounds examined and the effect of each upon the growth of this micro-organism.

TABLE 1. GROWTH OF *Bacillus cereus* IN THE PRESENCE OF VARIOUS PYRIMIDINE ANALOGUES

(Growth after time of addition of analogue as measured by optical density increase in 1 hr)

Analogue	Final concentration (μg ml)	Growth inhibition (%)
Thiouracil	130	0
Thiouracil	260	0
Isobarbituric acid	150	0
Isobarbituric acid	300	0
Nitroorotic acid	240	0
6–Azauracil	120	72
5–Fluorouracil	1·8	40
5–Fluorouracil	7·2	61
5–Fluoro-orotic acid	340	65
Bromouracil	300	0

In addition to 6-azauracil, the only analogues to produce an inhibition of growth of this organism were 5-fluorouracil and 5-fluoro-orotic acid. Investigations by Cohen *et al.*[13] using *E. coli* indicate that 5-fluorouracil interferes with the synthesis of nucleic acids. The metabolism of nucleic acids in *B. cereus*, also, is affected by this analogue. These observations will be published at a later date.

A more intensive study has been made on the effect of 6-azauracil on the meta-

bolism of *B. cereus*. Fig. 1(b) shows the inhibitory effect of 6-azauracil (120 μg/ml) upon growth. If the data are plotted on semilog paper a break in the curve may be observed in the culture which contains the pyrimidine analogue. This fact would suggest that the effect of the 6-azauracil is being reduced as growth proceeds.

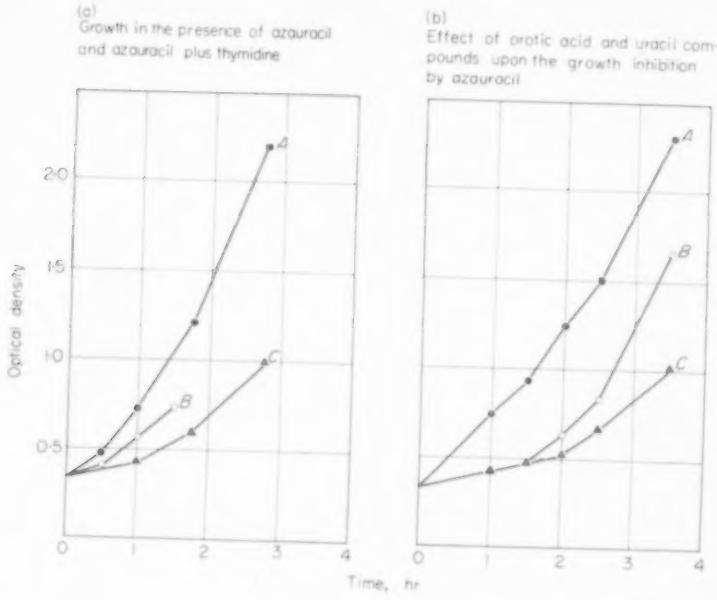

FIG. 1(a). A. Growth in the control culture. B. Growth in the presence of azauracil (120 μg/ml) and thymidine (450 μg/ml). C. Growth in the presence of azauracil (120 μg/ml).

FIG. 1(b). A. Growth in the control culture. B. Growth in the presence of azauracil (120 μg/ml) and uracil (120 μg/ml) uridine (200 μg/ml) or uridylic acid (260 μg/ml). C. Growth in the presence of azauracil (120 μg/ml) or azauracil (120 μg/ml) and orotic acid (130 μg/ml).

Synthesis in the presence of 6-azauracil

(a) *Cell wall.* The formation of cell wall material was followed in the presence of 120 μg/ml of 6-azauracil. The cell wall of *B. cereus* contains hexosamines, alanine, glutamic acid, diaminopimelic acid[14] and aspartic acid.[15] Cell wall synthesis was followed by determining the hexosamines[9] in the acid insoluble material. Azauracil was observed to suppress the synthesis of this component in the acid insoluble material (see Table 2).

TABLE 2. FORMATION OF CELL WALL MATERIAL IN THE PRESENCE OF AZAURACIL

Time (min)	Hexosamine (μg) in acid insoluble material per ml of bacterial suspension	
	No azauracil added	Azauracil (120 μg) added per ml at zero time
0	32	28
30	38	32
60	49	35
90	67	42

TABLE 2(A). FORMATION OF CELL WALL MATERIAL IN THE PRESENCE OF AZAURACIL AND THYMIDINE

Time (min)	Hexosamine (μg) in acid insoluble material per ml of bacterial suspension	
	Control and 400 μg thymidine added per ml	Azauracil (120 μg) added and 400 μg thymidine per ml at zero time
0	24	26
30	45	41
60	57	52
90	80	68

(b) *Effect of azauracil on the formation of RNA and DNA.* Figs. 2(a) and 3(a) illustrate the effect that azauracil has upon the formation of nucleic acids in *B. cereus*. The more pronounced effect is upon the formation of ribonucleic acid. If a lower concentration of azauracil (60 μg/ml) is used the percentage inhibition of synthesis of the two nucleic acids remains high. Furthermore, the inhibition of nucleic acid formation is measureable at least 30 min after the beginning of the incubation of the culture with the analogue.

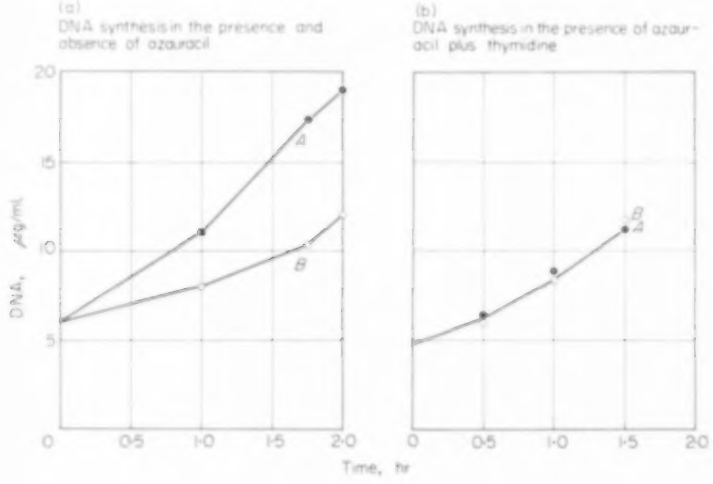

FIG. 2(a). A. DNA formation in control culture. B. DNA formation in the presence of azauracil (120 μg/ml).

FIG. 2(b). A. DNA formation in the presence of thymidine (450 μg/ml). B. DNA formation in the presence of azauracil (120 μg/ml) and thymidine (450 μg/ml).

(c) *Protein formation in the presence of 6-azauracil.* As in the case of the nucleic acids, protein formation also was inhibited when the bacteria were incubated in the presence of azauracil. Fig. 4(a) illustrates the extent of the inhibition produced by this pyrimidine analogue.

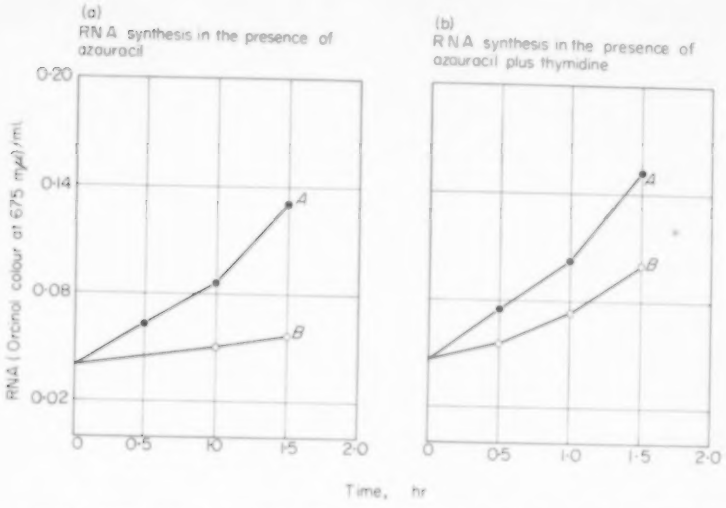

FIG. 3(a). A. RNA formation of control culture. B. RNA formation in the presence of azauracil (120 μg/ml).

FIG. 3(b). A. RNA formation in the presence of thymidine (450 μg/ml). B. RNA formation in the presence of azauracil (120 μg/ml) and thymidine (450 μg/ml).

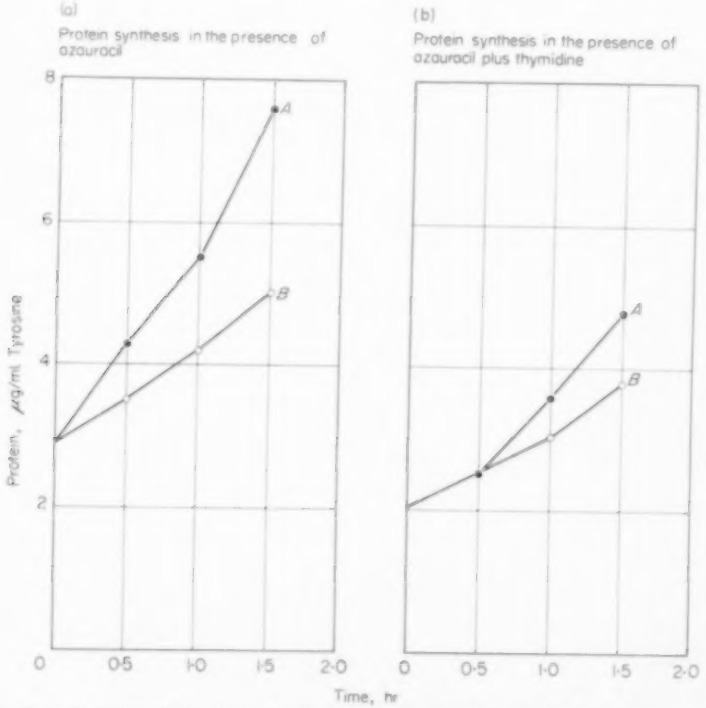

FIG. 4(a). A. Protein synthesis in control culture. B. Protein synthesis in the presence of azauracil (120 μg/ml).

FIG. 4(b). A. Protein synthesis in the presence of thymidine (450 μg/ml). B. Protein synthesis in the presence of azauracil (120 μg/ml) and thymidine (450 μg/ml).

Influence of naturally occurring pyrimidines upon the inhibition produced by azauracil

Fig. 1(b) shows the influence of uracil, uridine and uridylic acid upon the growth inhibition resulting from treatment with azauracil. Immediately after addition of one of these compounds the inhibitory effect of azauracil is reduced. It was observed, furthermore, that the addition of orotic acid, a precursor of uracil, to a culture inhibited by the analogue produced no reduction of inhibition. This observation suggested that a mechanism involved in the conversion of orotic acid to uracil was being inhibited by azauracil. Another explanation, however, was possible, namely that orotic acid was unable to gain entrance to the cell. Consequently the following experiment was performed in order to determine which of the two above explanations was valid. Suspensions of bacteria were incubated in the presence of either 2-C-14 uracil or 2-C-14 orotic acid with and without 6-azauracil. Samples were removed at various time intervals and added to an equal volume of cold 10% TCA containing a small quantity of the unlabelled precursor. The samples were allowed to stand in the cold for 30 min and then were filtered through a millipore filter and washed five times with 5% TCA, containing a small quantity of the unlabelled compound. Table 3 indicates

TABLE 3. INCORPORATION OF URACIL 2-C-14 AND OROTIC ACID 2-C-14 INTO THE NUCLEIC ACIDS OF *Bacillus cereus* IN THE PRESENCE OF AZAURACIL

Time (min)	Total counts/min			
	Labelled orotic acid	Labelled orotic acid plus azauracil 120 μg/ml	Labelled uracil	Labelled uracil plus azauracil 120 μg/ml
20	820	120	510	570
40	1845	285	1350	1500
60	2940	674	2830	3050

Conditions: 0·6 ml uracil 2-C-14 (0·15 μc/ml and 240 μg/ml); 0·6 ml orotic acid 2-C-14 (0·33 μc/ml and 54 μg/ml).

that orotic acid does enter the bacteria and that its incorporation into acid insoluble material is inhibited by azauracil to a much greater extent than is the incorporation of labelled uracil. A recent communication by Handschumacher[16] has stated that in *E. coli* the azauracil exerts its effect through the action of its riboside on orotidine decarboxylase. The action of azauracil may be similar in the case of *B. cereus*.

Partial reversal of azauracil inhibition by thymidine

Since both RNA and DNA formation were suppressed in the bacteria treated with azauracil an attempt was made to reverse the inhibition of the formation of DNA and then observe whether the synthesis of cell wall material, as measured by hexosamine content, still was reduced. It was of interest to know whether a direct relationship exists between RNA formation and cell wall formation. Since uracil production was inhibited by the presence of azauracil the production of thymidine for DNA synthesis would, as a result, be prevented. With this fact in mind thymidine was added in an attempt to reverse the DNA inhibition solely. Addition of thymidine (but not thymine) reduced the magnitude of growth inhibition (see Fig. 1(a)). At the same time DNA formation could be restored to normal while RNA synthesis although still

inhibited (see Figs. 2(b) and 3(b)) was increased. Two explanations suggested themselves as a result of this experiment: (1) thymidine produces a "sparing" action on the uracil available for total nucleic acid synthesis or (2) thymidine is converted into other pyrimidine components, such as uracil or uridine, necessary for ribonucleic acid synthesis.

The second possibility was tested in the following manner: labelled uracil was added to a culture of *B. cereus* growing in logarithmic phase; to a second culture labelled uracil plus thymidine were added. Samples were removed at various time intervals and added to an equal volume of cold 10% TCA and filtered through millipore filters following the procedure outlined above. The results of Table 4 indicate that the incorporation of labelled uracil is reduced by the presence of thymidine. A dilution of labelled uracil incorporation was produced also by the addition of cytidine

TABLE 4. INCORPORATION OF URACIL 2-C-14 INTO THE NUCLEIC ACIDS OF *Bacillus cereus* IN THE PRESENCE OF THYMIDINE AND CYTIDINE

Time (min)	Total counts/min		
	Labelled uracil	Labelled uracil 450 μg thymidine added per ml	Labelled uracil 450 μg per ml cytidine added
15	3019	1860	393
30	8143	3620	588
60	24,400	7544	901

Uracil 2-C-14 (3 μc/ml and 74 μg/ml)-0·3 ml added to 21 ml of bacterial suspension. Incubated at 30° C.

under the same conditions. In the latter instance oxidative deamination of the cytosine moiety probably takes place. Cytidine is a more efficient diluant of the labelled uracil than is thymidine a fact which suggests that the pathway to uracil (or uracil containing compounds) from thymidine is not favoured. The assumption that the greater part of the uracil enters RNA is justified in view of the relative amounts of the two nucleic acids in bacteria. Ribonucleic acid represents about 89 per cent of the total nucleic acid in *B. cereus* cell (H. Chantrenne, personal communication).

Effect of thymidine on protein and cell wall formation in azauracil-treated B. cereus

Fig. 4(b) indicates that under conditions such that DNA synthesis is restored to normal, by the addition of thymidine, but RNA synthesis is still inhibited, protein formation is inhibited. Furthermore, cell wall formation, as measured by hexosamine content of acid insoluble material, also was slightly lower than normal. The addition of thymidine to a culture treated with azauracil has resulted in a production of cell wall hexosamine which is greater than in a culture treated with azauracil alone. This result may now be explained in the light of the ability of the cell to produce uracil or a uracil derivative from thymidine. From the above studies it is impossible to deduce whether the formation of uridine nucleotides alone is essential for cell wall formation or whether RNA synthesis is also an essential factor. It should be realized that the effect of azauracil upon the production of cell wall hexosamine may be a secondary effect resulting from a lowered metabolic activity of the cell. Furthermore azauracil treated *B. cereus* observed under a microscope are indistinguishable from a normally growing culture.

DISCUSSION

The results presented in this paper are in agreement with the recent investigations of Handschumacher[16] who observed that a culture of *E. coli* treated with azauracil accumulate large amounts of orotic acid and orotidylic acid. His results showed, furthermore, that mice bearing L-1210 and L-5178 lymphomas when treated with azauracil in their drinking water accumulate orotic acid or orotic acid riboside (orotidine) in the acid soluble fraction of the tumours. The studies by Handschumacher[17] have suggested that orotidylic acid decarboxylase is the primary site of action of this pyrimidine analogue. In our investigations with *B. cereus* an inhibition of the incorporation of labelled orotic acid has been observed in the presence of azauracil without any effect upon the incorporation of labelled uracil. Furthermore, the fact that uracil, and not orotic acid, restores the growth of *B. cereus* treated with azauracil points to the site of action as being between orotic acid and uracil. In effect the addition of azauracil to *B. cereus* produces a culture which is uracil deficient.

The metabolic disturbance in nucleic acid production is not surprising in view of the lack of uracil. The formation of RNA is affected to the larger degree. It seems reasonable that the defect lies in the rate of production of RNA. The RNA formed in the presence of azauracil probably is normal with respect to purine and pyrimidine ratios. The nucleotides measured in the RNA determination are mainly purine nucleotides and these are reduced in amount in azauracil treated bacteria as are the uridine nucleotides.

The fact that the hexosamine content of the acid insoluble material decreases during incubation with azauracil may be a secondary effect resulting from a decreased formation of proteins necessary for the cell wall synthesizing systems. If the active compound in the inhibition of orotidine decarboxylase is azauridine as has been reported,[17] then it would seem that this component does not interfere with the function or formation of the uridine nucleotides necessary for the synthesis of cell wall material. The observation that *B. cereus* incubated with azauracil retain their rod-shaped appearance would lead us to believe that the decreased hexosamine formation is a reflection of the lowered metabolic activity of the cell. A paper by Takagi and Otsuji[2] has reported that *E. coli* under the influence of azauracil accumulate an N-acetylglucosamine ester to the extent of from five to six times the normal concentration. There have been no reports, however, on the appearance or characteristics of the cell wall of these treated bacteria.

Using thymidine it has been possible to reduce the inhibition of growth of the bacteria treated with azauracil. With the concentrations employed DNA formation is restored to normal and RNA formation, although still inhibited, is greater than in the absence of added thymidine. The influence of thymidine on the partial restoration of growth and RNA production may be due to the sparing action of thymidine on uracil for total nucleic acid formation or on the other hand it may be the result of a partial conversion of thymidine to uracil or a uracil derivative. To test the latter hypothesis the incorporation of C-14-uracil into *B. cereus* was measured in the presence and absence of thymidine. In these experiments the incorporation of uracil was inhibited to an extent of about 70 per cent. Although the thymidine may replace some of the uracil which would eventually enter the DNA as thymidine this sparing action cannot explain the total effect of the uracil dilution and the deduction must be made that part of the thymidine is converted into uracil or a uracil derivative. In the presence of

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azauracil the amount of uracil present in the bacteria is reduced, consequently the diversion of thymidine to uracil might be expected to be favoured. In this connexion a communication by Amos and Magasanik[18] has reported the conversion of uridine to thymidine, in bacteriophage, without apparent rupture of the pyrimidine sugar bond. A reverse of this reaction may be operative in *B. cereus* and may be favoured in the conditions of our experiment. In animal tissues, however, thymidine seems to be used exclusively for DNA formation with negligible amounts entering ribonucleic acid.[19, 20] Finally it should be added that the meaning of specific precursor in the case of thymidine should not be taken too literally. While thymidine may be a specific DNA precursor under certain physiological conditions and in certain experimental materials the view that it is specific in all instances may be questioned.

Under conditions, in the above experiments, in which DNA formation is normal and RNA formation is reduced the synthesis of protein takes place at a reduced rate. The relation between RNA and protein synthesis has been dealt with recently by Brachet[21] and by Chantrenne[22] in sufficient detail as not to warrant further discussion here.

A final comment should be made concerning the effect which thymidine has on diluting the incorporation of labelled uracil into the nucleic acids of *B. cereus*. The possibility exists that this dilution may be the result of a trace amount of uracil or a uracil containing compound in the thymidine sample. This possibility is now being investigated.

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INHIBITION OF MONOAMINE OXIDASE BY 1-PHENYLETHYLAMINES

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Abstract—Alkyloxy with or without additional alkyl or hydroxy substituents in the benzene ring considerably enhanced the weak anti-monoamine oxidase activity of 1-phenylethylamine. 1-(3-*n*-Hexyloxy-4-hydroxy-5-*n*-propylphenyl) ethylamine had good inhibitory power at 10^{-4}M at pH 6·8, but the activity declined on the alkaline side of neutrality. It antagonized the prolongation of barbiturate narcosis in mice due to reserpine.

BENZYLAMINE can be oxidized by monoamine oxidases, notably by an enzyme in horse serum,[1] but its derivatives are rarely mentioned as inhibitors of this class of enzyme.[2] By analogy with amphetamine, introduction of a methyl group to give 1-phenylethylamine derivatives could provide monoamine oxidase inhibitors. A range of substituted 1-phenylethylamines and observations on their overall effects in rats and mice were available and current interest in monoamine oxidase prompted their examination as possible inhibitors of these enzymes. The object was to explore the possibility of finding a potent *reversible* inhibitor and to look for any correlation between inhibitory potency and overall behavioural change or toxic effect in rats and mice.

MATERIALS AND METHODS

Amines. Most of these were remainders from previous studies.[3, 4]

x-*Bromo*-1-(3-n-*hexyloxyphenyl*) *ethylamine.* This amine precipitated on addition of one molecular proportion of bromine in 10% hydrochloric acid to cold aqueous 1-(3-*n*-hexyloxyphenyl) ethylamine hydrochloride. It crystallized from water, m.p. 154°C (Found: C, 50·1; H, 6·9; N, 4·2. Calc. for $C_{14}H_{23}ONBrCl$: C, 49·8; H, 6·6; N, 4·1%). Orientation of the firmly bound bromine atom was not investigated following unfavourable biological results.

1-(*p*-*n*-Alkyloxyphenyl)-2-aminopropanes (prepared by Mr. G. K. Ruffell). Equimolar quantities of *p*-hydroxybenzaldehyde and alkyl bromide in boiling alcohol treated by dropwise addition of alcoholic potassium hydroxide gave the corresponding *p*-alkyloxybenzaldehydes in 67–80 per cent yield. These were condensed with nitroethane[5] and the nitropropenes (40–50 per cent yield) either after crystallization from methanol or as the crude reaction product, were reduced with lithium aluminium hydride (5 molar proportions) in ether.[6] The bases were isolated by conventional methods and crystallized as the *hydrochlorides* or *hydrogen succinates*.

Structural Formula	R	Melting or boiling point	n_D^{20}	Formula	Found			Required		
					C	H	N	C	H	N
	n-Butyl	178°/25 mm*	1·5399							
	n-Butyl	178°/25 mm*	1·5340	$C_{11}H_{14}O$	74·1	8·0	—	74·1	7·9	—
	n-Pentyl	165–8°/15 mm	1·5296	$C_{12}H_{16}O_2$	75·0	7·9	—	75·0	8·4	—
	n-Hexyl	178–186°/16 mm	1·5230	$C_{13}H_{18}O_2$	75·1	8·2	—	75·7	8·8	—
	n-Heptyl	195–6°/17 mm	1·5214	$C_{14}H_{20}O_2$	76·4	10·0	—	76·3	9·2	—
	n-Octyl	127–8°/0·06 mm		$C_{15}H_{22}O_2$	76·8	9·0	—	76·9	9·5	—
	n-Butyl	55–57·5°	—	$C_{12}H_{17}NO_2$	65·9	7·0	5·8	66·4	7·3	6·0
	n-Octyl	50–51°	—	$C_{17}H_{28}NO_2$	70·4	8·5	4·6	70·1	8·7	4·8
	n-Butyl	Oxalate, 140–142°	—	$C_{12}H_{17}NO$† $C_2H_2O_4 \cdot H_2O$	57·1	7·6	4·4	57·1	8·0	4·4
	n-Pentyl	Succinate, 132–134°	—	$C_{13}H_{19}NO$ $C_4H_6O_4$	63·5	8·3	4·2	63·7	8·6	4·1
		Hydrochloride, 202–4° (softens at 148°)	—	$C_{13}H_{19}NO \cdot HCl$	65·0	9·2	5·5	65·4	9·3	5·5
	n-Hexyl	Succinate, 129–131°	—	$C_{14}H_{21}NO$ $C_4H_6O_4$	64·6	9·1	4·0	64·6	8·8	4·0
	n-Heptyl	Succinate, 129–131°	—	$C_{15}H_{23}NO$ $C_4H_6O_4$	64·9	9·0	3·9	65·4	9·1	3·8
	n-Octyl	Succinate, 127–128°	—	$C_{16}H_{25}NO$ $C_4H_6O_4$	66·4	9·0	3·7	66·1	9·3	3·7
		Hydrochloride, 224° (softens at 137°)	—	$C_{16}H_{25}NO \cdot HCl$	68·1	10·0	—	68·2	9·7	—

* Stoermer and Wodarg[7] quote b.p. 285°C.
† Loss at 100°C, 4·8 per cent.

p:*p*′-1-Aminoethyldiphenylether dihydrochloride was supplied by my colleague, Dr. F. C. Copp and Iproniazid (Marsilid) by Dr. A. Cohen, Roche Products, Ltd.

Assay. Though the method is not as accurate as others available, conventional manometry under oxygen at 37 °C was convenient for screening. Tryptamine (0·01 M) was the substrate. An acetone-dried powder[8] of guinea-pig liver (40 mg per flask) suspended in 0·067 M phosphate buffer provided the enzyme. A pH of 6·8 was chosen instead of the more usual value of 7·4 to minimize precipitation of the bases. Each amine, with a pre-incubation time of 10–15 min, was tested in duplicate at 10^{-3} M final concentration and inhibition per cent was calculated from the rate of oxygen uptake, corrected for blank, during 45 min. Those amines with notable activity were retested in duplicate at a range of concentrations.

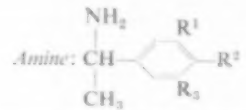
Effects of injection into mice of 1-(3-n-*hexyloxy*-4-*hydroxy*-5-n-*propylphenyl*) *ethylamine*. Groups of three mice were given either saline or the above amine (15 mg/kg) intraperitoneally. Their brains less cerebella, and livers were chilled, weighed, dispersed in cold phosphate buffer at pH 7·4 and dialysed at 0 °C overnight before manometric assay. Liver powder suspensions were also dialysed after inhibition by the amine and examined for removal of the inhibition.

The effect of the amine (25 mg/kg) intraperitoneally on the duration of barbiturate narcosis,[9] with or without reserpine pretreatment, was kindly tested by Mr. A. F. Green of these Laboratories in groups of five mice. In similar series, Iproniazid (100 mg/kg) was substituted for the amine.

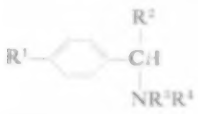
RESULTS

Approximate inhibitory potencies of twenty-eight amines are summarized in Table 1. In the homologous series of 1-(*p*-alkyloxyphenyl) ethylamines, activity reached a

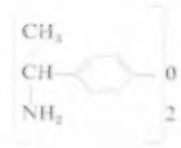
TABLE 1. INHIBITION OF MONOAMINE OXIDASE BY 1-PHENYLETHYLAMINES



R^1	R^2	R^3	Concentration giving 50 or 80% inhibition ($\times 10^{-4}$ M) 50	80	LD_{50} in mice (mg/kg)
H	OC_2H_5	H	> 10	—	180
H	$OC_3H_7^i$	H	> 10	—	178
H	$OC_3H_7^n$	H	10	—	—
H	$OCH(C_2H_5)_2$	H	> 10	—	140
H	$OC_4H_9^n$	H	2	5	170
H	$OC_5H_{11}^i$	H	2	5	—
H	$OC_5H_{11}^n$	H	1	3	108
H	$OC_6H_{13}^n$	H	2	5	76
H	$OC_7H_{15}^n$	H	—	10	80
H	OC_6H_{11}*	H	4	10	178
H	OC_5H_9†	H	10	—	172
H	OC_6H_5	H	2	5	97
$OC_6H_{13}^n$	H	H	2	4	—
OH	OC_6H_{11}*	H	3	10	250
OC_6H_{11}*	OH	H	1	5	180
$OC_6H_{13}^n$	OH	$C_3H_7^n$	—	1	57
$OC_2H_5^i$	OH	$C_3H_7^n$	10	—	—
H	OC_6H_{11}*	CH_3	4	10	80
H	OC_5H_{11}*	C_2H_5	1	6	—
H	OC_6H_{11}*	$C_3H_7^n$	3	—	48



R^1	R^2	R^3	R^4			
OC_2H_5	CH_3	CH_3	H	2	5	—
OC_2H_5	CH_3	CH_3	CH_3	1	10	104
OC_2H_5	CH_3	C_2H_5	H	1	4	—
OC_2H_5	CH_3	C_2H_5	C_2H_5	5	—	92
OC_6H_{11}	C_2H_5	H	H	7	—	104



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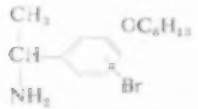
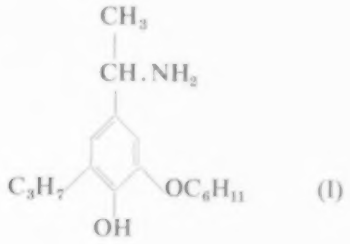


3 10 —

* C_6H_{11} = *cyclo*hexyl.
† C_5H_9 = *cyclo*pentyl.
1-Phenylethylamine inhibited 30 per cent at 10^{-2} M.

peak at *n*-pentyl and then rapidly declined. Members of the series above *n*-heptyl were insoluble in the buffer. Chain branching was not beneficial and activity corresponded to that of the longest straight chain present. *cyclo*-Alkyl ethers were less active than the corresponding *n*-alkyl ethers. The phenyl ether had considerable activity. *Meta* orientation appeared to make little difference though transposition of *cyclo*-hexyloxy and hydroxy between *meta* and *para* positions indicated a greater activity associated with the *meta* ether. Dialkylation of the amino group was deleterious but monoalkylation up to ethyl had little influence. Lengthening aminoethyl to 1-aminopropyl reduced activity in the instance studied. Introduction of further alkyl or hydroxy groups was beneficial and this was particularly evident in 1-(3-*n*-hexyloxy-4-hydroxy-5-*n*-propylphenyl)-ethylamine (I).



(I)

Some attempts to construct more active amines, using published work and the foregoing structure–activity relations as a guide, were unsuccessful. Thus, though halogen slightly increased the potency of *iso*-thioureas,[11] and also conferred activity in other series (unpublished observations), a nuclear bromine atom reduced the activity of 1-(3-*n*-hexyloxyphenyl) ethylamine. Introduction of a *p*-alkyloxy substituent (butyl to octyloxy) into amphetamine had little influence.

Experiments with 1-(3-n-*hexyloxy*-4-*hydroxy*-5-n-*propylphenyl*)*ethylamine.* This amine (I) had good inhibitory potency and was subjected to more detailed study within the limits of available material.

Influence of pH on inhibition in vitro. The amine (I) inhibited oxidation of tryptamine, 5-hydroxytryptamine, tyramine and hydroxytyramine at concentrations above 10^{-4} M with buffer pH6·8. There was slight activity at 10^{-6} M. Under similar conditions, inhibitions due to (I) and to Iproniazid at 5×10^{-5} M were, respectively, 85, 94 per cent and 46, 37 per cent. The amine (I) lost potency as the pH rose towards the optimum for the enzyme and was about 70 per cent for the same concentration at pH 7·0. Though the active species of phenylethylamines may be the ion[2] the pK_a of 7·2 (in 25 per cent alcohol at 37 °C) suggests that the fall in activity is more related to increasing activity of the enzyme as it approaches its optimum.

Persistence of inhibition in vitro *but not* in vivo. As observed withIpron iazid[12] and Pentamidine,[10] inhibition due to the amine (I) *in vitro* was difficult to remove by dialysis or washing. Washing the inhibited enzyme four times with phosphate buffer reduced inhibition from 83 to 69 per cent. Colorimetric estimation by the method of Brodie and Udenfriend[13] showed that there was a concentration of the amine onto the liver particles (Table 2) and the low total recovery when the initial concentration exceeded 2×10^{-5} M suggested that a part of the amine became firmly bound to the

particles. Nevertheless homogenates of liver and brain prepared from dosed mice showed no impairment of monoamine oxidase activity after dialysis overnight. Dialysed liver homogenates prepared from mice at 1, 3 and 18 hr after doses of 15 mg/kg amine intraperitoneally consumed 178, 188 and 185 μl O_2/g tissue per hr,

TABLE 2. DISTRIBUTION OF 1-(3-*n*-HEXYLOXY-4-HYDROXY-5-*n*-PROPYLPHENYL) ETHYLAMINE IN A LIVER POWDER SUSPENSION

Initial concentration (M)	Final concentration in solution (μg ml)	Concentration in liver particles (μg g)	Total recovery (%)
10^{-4}	8·1	5780	—
10^{-4}	9·7	5400	57
5×10^{-5}	4·1	2890	65
2×10^{-5}	4·1	550	102

40 mg liver powder homogenized in 10 ml 0·06 M phosphate buffer pH 6·8 and shaken for 30 min at room temperature. Total recovery refers to amine found after adding excess HCl before centrifuging the tissue debris.

respectively, compared to a control value of 180 μl/g per hr while oxidizing tryptamine. Brain homogenates gave a similar negative result.

Antagonism of reserpine-induced prolongation of barbiturate narcosis. Table 3 shows that whereas Iproniazid (100 mg/kg) effectively prevented the prolongation of barbiturate narcosis due to reserpine at 24 hr after the dose, the amine (I) (25 mg/kg = half

TABLE 3. INHIBITION OF PROLONGATION OF BARBITURATE NARCOSIS DUE TO RESERPINE IN MICE BY 1-(3-*n*-HEXYLOXY-4-HYDROXY-5-*n*-PROPYLPHENYL) ETHYLAMINE

Hours before barbiturate		Mean recovery time (min)		Deaths
		Sitting	Walking	
28 hr	4 hr			
Saline	Saline	36	45	0
Saline	Reserpine	114	> 128	0
Iproniazid	Saline	31	37	1
Iproniazid	Reserpine	41	48	2
Amine	Saline	53	74	0
Amine	Reserpine	121	> 136	0
4 hr	2 hr			
Saline	Saline	36	45	0
Reserpine	Saline	156	> 186	0
Amine	Saline	44	50	0
Amine + reserpine	Saline	65	76	1
Amine + reserpine	Amine	74	> 96	0

Groups of five mice received drugs at times stated before sodium pentobarbital (60 mg/kg i.v.). Doses were: reserpine: 2·5 mg/kg i.p.; Iproniazid: 100 mg/kg i.p.; amine; 25 mg/kg i.p.

the LD_{50} dose) under similar conditions was ineffective. When the amine was given shortly before reserpine there was a marked antagonism confirming the previous finding that its inhibitory action on monoamine oxidase is readily reversible *in vivo*.

DISCUSSION

The beneficial influence of alkyl chains on anti-monoamine oxidase activity in 1-phenylethylamines finds parallel with observations on mono-amidines[10] and *iso*-thioureas.[11] It has been shown that primary aliphatic amines are most readily oxidized at C_5 to C_6[2] and that higher alcohols inhibit. Nevertheless the anti-monoamine oxidase activity of amphetamine[14] was reduced and analeptic motor activity in mice almost abolished by introduction of those *para*-alkyloxy substituents that considerably enhanced the activity of 1-phenylethylamine.

Though the increase in inhibitory potency due to substitution in 1-phenylethylamines was accompanied by increase in toxicity to mice (Table 1), this was not unfavourable from "therapeutic index" considerations.

There was no indication that inhibitory potency in the series paralleled either analeptic,[14, 15] convulsant or depressant activity. None of the amines induced the motor activity in mice characteristic of amphetamine and few induced morphine-like hyper-irritability. Rather they depressed spontaneous movement at moderate dosage, becoming persistent convulsants at high dosage with no clear distinction between clonic or tonic type. The most active member of the series was, however, clearly distinguished from the rest by virtual lack of convulsant activity. At moderate dosage it tended to produce a long lasting depression. Comparison with Iproniazid in the barbiturate narcosis test suggests that this amine can inhibit monoamine oxidase *in vivo* at relatively low doses. It is interesting that its activity is greater at slightly acid pH following an assessment of published work that biological fluids may often have a pH value nearer 6·8 than 7·4.[16]

Acknowledgements—Mr. A. F. Green has kindly permitted inclusion of his results in the barbiturate narcosis test. Grateful acknowledgement is made to Dr. F. C. Copp, Dr. A. Cohen and Mr. G. K. Ruffell for provision of materials, and to Mr. P. R. W. Baker for the analyses and to Messrs. R. A. Nightingale and D. W. G. Richardson for technical assistance.

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ISOLATION OF BUFOVIRIDINE FROM THE SKIN OF *BUFO VIRIDIS* AND ITS IDENTIFICATION AS DIHYDROBUFOTHIONINE

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Abstract—A procedure for the isolation of bufoviridine from skin extracts of the green toad *Bufo viridis* is described, as well as the properties of the crystalline base. Bufoviridine is shown by elementary analysis and by the study of some of its derivatives to be dihydrobufothionine, viz. the O-sulphate of bufotenine.

The general biological interest of the identification in the living organism of O-conjugation derivatives of hydroxyindoles is discussed.

THE skin of the green toad, *Bufo viridis*, contains, in addition to large amounts of bufotenine (N:N-dimethyl-5-hydroxytryptamine) and small amounts of 5-hydroxytryptamine, N-methyl-5-hydroxytryptamine and bufotenidine ((β-[5-hydroxyindolyl-(3)]-ethyl) trimethylammonium), a remarkable quantity of another hitherto unidentified indolealkylamine which was provisionally called *bufoviridine*.[1]

The new indole derivative can easily be distinguished on paper chromatograms from all the other known indole compounds of the amphibian skin by its R_F values and its colour reactions. This paper describes the isolation and chemical identification of bufoviridine, as well as some of its chemical and paper-chromatographic properties.

EXPERIMENTAL

Isolation of bufoviridine

Some 2100 specimens of *Bufo viridis* were collected near Parma (North Italy) in June 1956 and June 1957. The fresh skins of the animals, including parotoid glands, weighed 4600 g. They were extracted, for from 3 to 5 days, with 4 parts (w/v) of 99% acetone, and then re-extracted, for another 3–4 days, with 3–4 parts of 80% acetone. The combined filtrates were kept in the refrigerator, in dark bottles, for 6–18 months.

Twenty-eight litres of the acetone extract, corresponding to 4 kg of fresh skin, were concentrated by distillation under reduced pressure, at 40–45 °C, to about 2 l. A brownish resinous material which was found to adhere to the bottom of the distillation flask was not further considered in this study.

The dark brown aqueous liquid was decanted and extracted repeatedly with light petroleum ether in order to remove fats. The distillation was then continued until the volume was about 500 ml. The resulting turbid liquid was divided into two portions, and each portion was extracted with peroxide-free ethyl ether in a Friedrichs automatic extraction apparatus for 48 hr. The aqueous phases became perfectly clear.

They were evaporated separately to dryness, under reduced pressure, and the residues were dissolved in 200 ml of absolute methanol. A precipitate containing only traces of indolealkylamines was formed and filtered off. The clear filtrates were then passed through two alumina columns, 3·3 cm in diameter. The alumina (aluminium oxide Merck, according to Brockmann) weighed 200 g and reached a height of 29 cm in the column. Each column was subsequently washed with 750 ml of absolute methanol and the effluent collected in four divided fractions of 200 ml each. All the fractions were examined by paper chromatography. Fraction (I) contained nearly all the bufotenine and bufotenidine and 75–80 per cent of the bufoviridine; fraction (II) contained traces of bufotenine, bufotenidine, 5-hydroxytryptamine and N-methyl-5-hydroxytryptamine as well as 20 per cent of the bufoviridine; fraction (III) contained only traces of 5-hydroxytryptamine and bufoviridine; no indolealkylamine was found in fraction (IV).

Although no separation of the different indolealkylamines was obtained by this procedure, a large amount of pigments and other impurities was retained by the alumina.

Fractions (III) and (IV) were discarded; fractions (I) and (II) were combined and the resulting mixture was evaporated to dryness at 40 °C and the residue dissolved, under continuous stirring, in 800 ml of boiling 96% ethanol. After a precipitate containing only traces of indolealkylamines had been filtered off, the clear liquid was divided into four portions which were submitted separately to a second absorption chromatography on aluminium oxide. In contrast to bufotenine and bufotenidine, bufoviridine was nearly completely absorbed in this way, and could be eluted only by 80% ethanol. The distribution of indolealkylamines in the eluates obtained with falling concentrations of ethanol will be seen from Table 1.

TABLE 1. ELUTION FROM AN ALUMINA COLUMN OF THE DIFFERENT INDOLEALKYLAMINES OCCURRING IN THE SKIN OF *Bufo viridis* BY MEANS OF FALLING CONCENTRATIONS OF ETHANOL

Eluates			Colour	Indolealkylamine content		
No.	% Ethanol	Volume (ml)		Bufotenine	Bufoviridine	Bufotenidine
1	96	200	dark brown	+++++	(+)	+
2	96	100	yellow	(+)	0	(+)
3	96	100	pale yellow	0	0	0
4	80	100	pale yellow	0	0	0
5	80	200	brown	0	+++	(+)
6	80	100	yellow	0	0	0
7	70	100	yellow	0	0	0
8	70	100	yellow	0	0	0
9	30	300	yellow	0	0	0

Eluates (2) and (5) contained also small amounts of N-methyl-5-hydroxytryptamine and 5-hydroxytryptamine, respectively.

It appears from Table 1 that almost all the bufoviridine was contained in eluate (5). Thus the combined eluates (5) were evaporated to dryness under reduced pressures and the residue, weighing approximately 2·5 g, was repeatedly washed with small amounts of boiling 99% ethanol (50 ml altogether). After drying, the ethanol-insoluble fraction

weighed 1·59 g. Additional bufoviridine-containing fractions were obtained at −10 °C by adding successively to the clear ethanol filtrate 50 ml of absolute ethanol (precipitate (1) = 0·15 g), 50 ml of peroxide-free ethyl ether (precipitate (2) = 0·274 g), and finally a further 150 ml of peroxide-free ethyl ether (precipitate (3) = 0·31 g).

Although all the precipitates and the final ethanol-ether liquid contained considerable amounts of bufoviridine, the bulk of the substance was present in the first ethanol-insoluble fraction and in precipitate (1). They were combined (1·74 g) and then dissolved in a small volume of boiling water (20 ml). On cooling, thick yellowish crystals of bufoviridine separated. After grinding and washing with 96% ethanol, which removes some colour, the white, somewhat sticky powder weighed 0·98 g. It was again dissolved in 20 ml of hot water. Absolute ethanol (100 ml) and peroxide-free ethyl ether (100 ml) were then gradually added. The turbid liquid after standing for a week at −10 °C in the refrigerator, yielded a copious crop of nearly colourless crystals, weighing 0·802 g. Part (0·4 g) of this material was recrystallized to a constant melting point from 80% ethanol + peroxide-free ethyl ether. The yield of pure bufoviridine base was 0·32 g.

The combined eluates (1) contained 2·55 g of bufotenine base (= 637μg/g fresh skin), as determined by the method of Udenfriend *et al.*,[2] and approximately 6 g of urea.

Properties of bufoviridine

Melting point. Bufoviridine when heated in the capillary at a rate of 2°C/min, melted with decomposition at 210–212 °C.

Empirical formula

Calc. for $C_{11}H_{16}O_4N_2S.H_2O$: C, 47·67; H, 6·00; O, 26·46; N, 9·27; S, 10·60%
Found for bufoviridine : C, 47·56; H, 6·22; O, 26·04; N, 9·06; S, 10·27%

Other properties. Bufoviridine is rather hygroscopic. Even if preserved in dessiccator over calcium chloride it slowly absorbs humidity and crystals tend to conglomerate. At the same time it slowly hydrolyses, giving origin to a derivative which was indistinguishable from bufotenine, by paper chromatography.

Bufoviridine is moderately soluble in hot water, and much more so in boiling water. It has, on the contrary, a very limited solubility in absolute ethanol (even boiling) and is almost insoluble in anhydrous ethyl ether.

Chromatographic behaviour and colour reactions

Table 2 shows the R_F values of bufoviridine with four different solvent systems, as compared with those of other naturally occurring indole derivatives. By spraying the chromatograms with an acid solution of diazotized *p*-nitroaniline, a pale pink colour developed, which gradually became more intense and, on alkalinization with a 5% solution of sodium carbonate, turned into a cherry-red colour; with diazotized sulphanilic acid, the colour was pale yellow, turning slowly to red in acid medium, and intense brick-red in alkaline medium; with the NNCD reagent[3] (2-nitro-2-chloro-1-diazobenzene-α-naphthalene sulphuric acid; 0·1–0·3% solution in 0·1 N HCl), a pale pink colour appeared at the beginning, turning gradually into a more intense peach-red.

TABLE 2. R_F VALUES OF BUFOVIRIDINE AND RELATED INDOLE DERIVATIVES WITH DIFFERENT SOLVENTS

(The ascending unidimensional technique on Whatman no. 1 paper was employed)

Compound	Solvent			
	Distilled water	5% KCl in dist. water	*n*-Butanol + acetic acid + water (4:1:5)	*n*-Butanol + 25% methyl-amine (8:3)
Bufoviridine	0·75	0·76	0·28	0·52
Bufotenine	0·25–0·30	0·50	0·50	0·84
Dehydrobufotenine	—	—	0·64	0·34
Bufotenidine	—	—	0·29	0·22
N-Methyl-5-hydroxytryptamine	—	—	0·48	0·79
5-Hydroxytryptamine	0·18–0·23	0·43–0·45	0·45	0·62
Tryptamine	0·29–0·35	0·56–0·59	0·63	0·89
5-Hydroxytryptophan	0·43–0·49	0·42–0·43	0·22	0·14
5-Hydroxyindoleacetic acid	0·83–0·87	0·59–0·60	0·81	0·16
5-Hydroxytryptamine-O-glucuronide*	—	—	0·13	0·12
Bufotenin-O-glucuronide*	—	—	0·18	0·27
Bufoviridine before acid hydrolysis	0·75	0·76	0·28	0·52
Bufoviridine after acid hydrolysis	0·38	0·68	0·48	0·85
Methylated derivative of bufoviridine before acid hydrolysis	—	—	0·20	0.02
Methylated derivative of bufoviridine after acid hydrolysis	—	—	0·27	0·20

* Partially purified from urines of rats given 5-hydroxytryptamine and bufotenine, respectively.

Table 3 shows the colour reactions given by bufoviridine with the three diazonium salts above (in acid medium), as compared with the reactions given by the other indole derivatives listed in Table 2.

TABLE 3. COLOUR REACTIONS OF BUFOVIRIDINE AND RELATED INDOLE DERIVATIVES

Compound	Diazotized *p*-nitroaniline	Diazotized sulphanilic acid	NNCD reagent
Bufoviridine	orange-red	pink (orange)	peach-red
Bufotenine	cherry-red	wine-red	peach-red
Dehydrobufotenine	orange	brown-red	orange-yellow
Bufotenidine	cherry-red	wine-red	peach-red
N-Methyl-5-hydroxytryptamine	cherry-red	wine-red	peach-red
5-Hydroxytryptamine	cherry-red	wine-red	peach-red
Tryptamine	orange-yellow	yellow	orange-yellow
5-Hydroxytryptophan	cherry-red	wine-red	peach-red
5-Hydroxyindoleacetic acid	cherry-red	wine-red	peach-red
Methylated bufoviridine (O-sulphate of bufotenidine)	orange-red	pink (orange)	brown-rose
O-Glucuronide of 5-hydroxytryptamine	yellow	yellow	orange
O-Glucuronide of bufotenine	yellow	yellow	orange

Other spraying reagents used in the characterization of bufoviridine were as follows: 1% potassium ferricyanide + ammonia vapours (pale yellow turning to a light pink yellow); 2% alcoholic solution of *p*-dimethylaminobenzaldehyde + HCl vapours

(light blue, deep blue); 2% alcoholic solution of dichloroquinonechlorimide (no colour in acid medium, progressively increasing violet colour in alkaline medium); ninhydrin (violet).

On untreated chromatograms run with the butanol:acetic acid:water mixture, the bufoviridine spot gave no fluorescence reaction; this gradually developed after spraying with sodium carbonate (bluish fluorescence), or with bichromate + formalin, according to Shepherd *et al.*[4] (greenish fluorescence).

It will be seen that bufoviridine is easily hydrolysed by strong acids, giving origin to bufotenine. Thus, it is rather difficult to decide whether colour reaction which gradually develop in acid medium are due exclusively to bufoviridine itself or, at least in part, also to bufotenine. Generally, the first alternative seems to be more probable.

Derivatives of bufoviridine

(a) Five milligrams of bufoviridine were dissolved in 2 ml N HCl and the solution was heated for 20 min in a boiling water bath. The substance was quantitatively transformed into a compound indistinguishable from bufotenine both by paper chromatography, using all the previously listed solvent systems and colour reactions, and by its pharmacological effects on the rat uterus preparation and on the blood pressure of the spinal cat. The quantitative estimation of bufotenine was carried out according to Udenfriend *et al.*[2] On adding a few drops of a solution of barium chloride to the solution of bufoviridine submitted to acid hydrolysis, a white turbidity of barium sulphate immediately developed. Barium chloride did not cause any turbidity when added to a neutral solution of bufoviridine.

(b) Five milligrams of bufoviridine were dissolved in 2 ml of water and the liquid was vigorously shaken after adding a few drops of methyl iodide and enough 2 N NaOH to produce an alkaline reaction. A methylated derivative of bufoviridine was thus obtained which possessed the same colour reactions as the parent substance and the R_F values shown in Table 2. Following acid hydrolysis it gave origin to a new derivative, which was both chromatographically and biologically (blood pressure of the spinal cat) indistinguishable from bufotenidine. Hence, the methylated derivative of bufoviridine was, in all probability, the O-sulphate of bufotenidine.

DISCUSSION

The results of elementary analysis and the study of bufoviridine derivatives leave no doubt that bufoviridine can be identified with the O-sulphate of bufotenine, viz. the dihydro-derivative of bufothionine. The name bufoviridine should therefore be dropped in favour of the chemical denomination *dihydrobufothionine*. So far, the

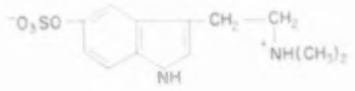

FIG. 1. Dihydrobufothionine (bufoviridine)

substance has been shown to occur in the skin of *Bufo viridis* and in that of *Bufo calamita*.

The O-conjugation of 5-hydroxyindoles with sulphuric acid seems to be not infrequent in the living organism. Bufothionine, found in the skins of *Bufo formosus*[5], *Bufo arenarum*[6] and *Acris crepitans*,[7] was the first example of this series of compounds. Another interesting example was represented by the O-sulphate of 5-hydroxytryptamine found in rat liver homogenates incubated with 5-hydroxytryptamine,[8] and in urine of rats given 5-hydroxytryptamine.[8b] It is to be expected that other O-sulphates of hydroxyindoles will be discovered in the future.

It is evident that esterification of the phenolic hydroxy group with sulphuric acid may represent a route for hydroxyindole metabolism. In some localizations of hydroxyindoles (e.g. amphibian skin) this metabolic route may be of considerable importance, in others only of limited interest. In the mammalian organism, for example, O-conjugation with glucuronic acid seems to be predominant, as shown by the fact that a considerable amount of administered 5-hydroxytryptamine and bufotenine is found in urine in the form of the corresponding O-glucuronides.[9, 10]

Quite recently it has been demonstrated that hydroxyindoles are capable of O-conjugating with another inorganic acid. We refer to the orthophosphoric acid, which has been found to esterify the phenolic hydroxy group of 4-hydroxy-N:N-dimethyltryptamine, giving origin to psilocybin, the hallucinogenic principle isolated by Hofmann *et al.*[11] from *Psilocybe mexicana*.

O-Conjugation with inorganic or organic acids is generally considered, even more than O-methylation, a way of inactivating highly active biogenic amines. This opinion seems to be correct in the majority of cases, but the recent example of psilocybin shows that great caution is necessary before any generalization can be made, as Hofmann and Troxler[12] succeeded in demonstrating that psilocin, 4-hydroxy-N:N-dimethyltryptamine, produced exactly the same vegetative and psychological effects as its phosphoric ester, psilocybin.

In so far as the amphibian skin is concerned, the significance of O-conjugation of bufotenine and dehydrobufotenine with sulphuric acid is completely obscure, since there are good reasons to believe that all the indolealkyamines in the cutaneous glands of amphibians are products indifferent to the animals, destined for external secretion.

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THE EFFECT OF SOME ORGANIC PEROXIDES ON SULPHYDRYL ENZYMES

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Abstract—Succinyl peroxide, a radiomimetic compound, has been found to be a powerful inhibitor of several —SH enzymes and with few exceptions to have much less effect on non —SH enzymes. The site of reaction of the peroxide with the enzymes is likely to be the —SH groups of the cysteine residues or the sulphur of the methionine residues, either or both of which may be oxidized. Interaction with certain other groups is not, however excluded.

INTRODUCTION

SEVERAL recent investigations have shown that certain organic peroxides are mutagenic, and further that peroxides may be important as destructive intermediates formed when radiation damages living organisms.

The first observation that organic peroxides had a mutagenic effect appears to have been made by Wyss *et al.*[1] Subsequently, Dickey *et al.*[2] clearly demonstrated that several peroxides had a mutagenic action on *Neurospora*. Loveless[3] showed that *tert.* butyl hydroperoxide caused chromosome breakage and in general possessed strong radiomimetic properties when tested on the roots of *Vicia faba*. More recently, Latarjet[4] studied the effect of succinyl peroxide and some other organic peroxides on bacteria, bacteriophage and transforming agent and found that the effects observed closely resembled those produced by radiation. Peroxides of fatty acids have also been shown to be highly toxic when injected into mammals.[5]

The organic peroxides form one of the most interesting groups of radiomimetic compounds since there is evidence of peroxide formation by X-rays in the living animal. Dubouloz and Dumas[6] showed that lipid peroxides were produced in the skin of the irradiated rat, and Horgan and Philpot[7] demonstrated peroxide formation in irradiated whole mice.

Very little is known concerning the biochemistry of the interaction of organic peroxides with proteins in general and enzymes in particular. Enzymes can be used under conditions which are similar to those prevailing physiologically and can frequently prove useful tools for the study of the reaction of various compounds with proteins. Furthermore, a study of enzyme activity in the presence of the compound under investigation is often a sensitive indicator of protein–compound combination. When an enzyme has been discovered which is strongly inhibited by the compound, the study of protective agents then makes possible an indirect investigation of the reactive groups of the enzyme which are especially sensitive to attack.

A study has thus been made of the effect of organic peroxides on several enzymes and this has included a detailed examination of sulphydryl enzymes. Succinyl peroxide

has been used in most experiments but in certain cases a comparison has also been made with benzoyl peroxide and hydrogen peroxide. The study of peroxides has been concentrated on the effects produced by dilute solutions (usually 10^{-3} M or more dilute) since it is these which must be of primary interest when considering modes of biological action.

Sulphydryl enzymes have been found to be most readily attacked by peroxides but certain other enzymes, not usually regarded as —SH were also found to be inactivated. A preliminary account of this work has already been reported.[8]

EXPERIMENTAL

Materials

Succinyl peroxide (O. CO. CH_2. COOH)$_2$ was a gift from Dr. Latarjet. A 0·01 M solution was prepared in water immediately before use and neutralized with NaOH (0·01 N). In aqueous solution the peroxide is immediately hydrolysed to per-succinic acid (HO.O.CO.CH_2.CH_2.COOH) which is the active principle.[4] It is not stable and loses activity at the rate of about 5 per cent per day.

Benzoyl peroxide was synthesized by the method described by Gatterman[9], and recrystallized 3 times from a mixture of 2 vols. methyl alcohol:1 vol. chloroform.

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Enzyme preparations and activity measurements

The methods of preparation and determination of the activity of most enzymes used have been previously described by Wills[10, 11]. Other methods were:

Pancreatic lipase. A purified enzyme was prepared as described by Wills[12].

Trypsin and Pepsin. These were used as 0·01% solutions of commercial crystalline preparations (Armour).

Proteolytic enzyme activity. In addition to the manometric method already described,[11] the turbidimetric method of Riggs and Stadie[13] was used with the substitution of denatured crystalline bovine serum albumin for egg albumin as substrate.

RESULTS

Effect of succinyl peroxide on enzymes

The effect of 10^{-3} M succinyl peroxide on eighteen different enzymes and some enzyme systems has been studied. The enzyme was usually left in contact with the peroxide for a standard period of 15 min before its activity was measured. All sulphydryl enzymes tested were found to be inhibited whilst most non-sulphydryl enzymes were unaffected (Table 1). In a very few cases, e.g. when trypsin and pepsin were used, a slight increase of enzyme activity was obtained. This has previously been observed in the case of pepsin.[4]

Enzymes shown in Table 1 as inhibited were not equally sensitive to peroxide. Urease was extremely sensitive, being 100 per cent inhibited by any solution of succinyl peroxide stronger than 10^{-5} M, and 85 per cent inhibited by 5×10^{-6} M. Most other enzymes included in this group were less affected; thus succinyl peroxide (10^{-3} M) caused 58 per cent inhibition of pancreatic lipase and 40 per cent inhibition of cholinesterase, both enzymes being unaffected by 10^{-4} M peroxide.

Enzymes of similar function from different sources sometimes showed quite different responses to succinyl peroxide. Thus malt amylases were powerfully inhibited

by peroxide but salivary and pancreatic amylases were resistant to high concentrations of peroxide.

TABLE 1. THE EFFECT OF 10^{-3} M SUCCINYL PEROXIDE ON SOME ENZYMES

Enzymes inhibited	Enzymes unaffected
Urease (SH)	Pepsin
Succinic dehydrogenase (SH)	Trypsin
Papain (SH)	Serum esterase
Pancreatic lipase (SH)	Salivary amylase
Cholinesterase (SH)	Pancreatic amylase
Triosephosphate dehydrogenase (SH)	Xanthine oxidase
β-Amylase (SH)	D-Aminoacid oxidase
α-Amylase	Cytochrome oxidase
Invertase	Carbonic anhydrase
Tyrosinase	

The effect of benzoyl peroxide and hydrogen peroxide on enzymes

Benzoyl peroxide and hydrogen peroxide were both found to be effective, but somewhat less efficient inhibitors of urease when used under conditions similar to those employed for succinyl peroxide. Both benzoyl and hydrogen peroxide in 10^{-4} M solutions caused about 80 per cent inhibition and 3×10^{-5} M solutions only 60 per cent inhibition. Succinyl peroxide completely inhibits urease at both these concentrations.

The effect of pH and composition of the buffer on succinyl peroxide inhibition

The majority of enzymes were examined at, or near their optimum pH. In the case of urease, however, most experiments were carried out at pH 5·5 since this is convenient for the manometric determination of activity. Using a Nesslerization technique the effect of peroxide on urease was examined over the pH range of 4·8–8·0 using acetate, citrate, tris-maleate and phosphate buffers. In general, succinyl peroxide was found to be more toxic to urease as the pH was made more acid but the effect was complicated by the type of buffer used. No significant differences in the inhibition produced by 2×10^{-5} M succinyl peroxide were detectable over the pH range 4·8–5·6 in acetate buffer, but in citrate buffer, although this same concentration of succinyl peroxide caused 100 per cent inhibition at pH5·6, the inhibition fell to 65 per cent when the pH was adjusted to 6·7. When tris-maleate buffer was used, however, the pH effect was much less marked and the inhibition produced by 5×10^{-5} M succinyl peroxide only declined from 100 per cent at pH 5·6 to 94 per cent at pH 8·0.

Phosphate is strongly inhibitory to urease and the addition of phosphate was found to protect urease against succinyl peroxide. In citrate buffer at pH 5·8, addition of $2{\cdot}5 \times 10^{-2}$ M sodium phosphate reduced the inhibition caused by 5×10^{-5} M succinyl peroxide from 100 to 30 per cent but this is largely due to an effect on the rate of the control. Grant and Kinsey[14] have previously shown that phosphate can protect urease against certain alkylating reagents.

Trypsin, which was previously reported to be unaffected by high concentrations of succinyl peroxide,[8] has now been found to be slightly affected in acid solutions of succinyl peroxide. After 30 min contact at pH 5·0, succinyl peroxide (10^{-3} M) caused 45 per cent inhibition of the enzyme although there was no effect if the pH was greater than 8·0, the pH at which the previous tests were made.

Effect of contact time on enzyme inhibitions

Most experiments were carried out using a contact period of 15 min for enzyme and peroxide before determining the enzyme activity. This contact period was varied with some enzymes and different responses were obtained (Fig. 1). The reaction of

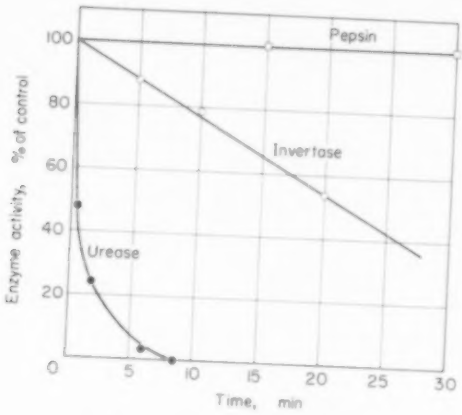

FIG. 1. The effect of contact time on enzyme inhibition by succinyl peroxide. Pepsin and invertase were treated with 10^{-3} M succinyl peroxide. Urease was treated with $3{\cdot}3 \times 10^{-5}$ M succinyl peroxide.

peroxide with —SH enzymes such as urease was very rapid and completed in a few minutes. Almost no effect was observed with pepsin in 30 min whilst invertase showed a steady and intermediate rate of fall of activity, although all three enzymes were exposed at the same pH. Over much longer time periods, e.g. 2-4 hr, pepsin has been found to lose a small percentage of its activity.[4]

Protection of crystalline urease against peroxide inhibition

The effect of a number of possible protective agents for urease was extensively investigated in order to try to establish the nature of the groups in urease and other enzymes which were attacked by peroxide.

The substance under test was mixed with succinyl peroxide in a buffer for a definite time, usually 5 min and then urease added. Any reduction in the inhibiting action of the peroxide was measured by determination of the enzyme activity. The concentration of succinyl peroxide used most frequently was $3{\cdot}3 \times 10^{-5}$ M, more than adequate to cause 100 per cent inhibition of urease. A reaction or combination of the test substance with peroxide was indicated by a reduction in the inhibition of urease.

Most proteins tested protected when present in adequate concentration (Table 2). Serum albumin was more effective than gelatin as a protective agent but it was necessary for the concentration of albumin to be about 125 times that of the succinyl peroxide on a weight for weight basis to give complete protection. The molecular ratio for complete protection was, however, very close to 2 moles succinyl peroxide:1 mole serum albumin and this may indicate that there are two reactive groupings in each mole of protein. Good protection was also obtained using native and denatured egg albumin, and peptide mixtures in the form of bacteriological peptone. Commercial preparations of thymus and yeast nucleic acids gave very weak protection of urease

TABLE 2. PROTECTION OF UREASE BY PROTEINS AGAINST INHIBITION BY SUCCINYL PEROXIDE

($3{\cdot}3 \times 10^{-5}$ M succinyl peroxide (0·0078 g./l) in contact with protein for 5 min before addition of urease).

Protein	Conc. (g/l.)	Protection (%)
Serum albumin	5	100
	1	100
	0·3	0
Gelatin	2	82
	1	24
	0·3	0

against succinyl peroxide. No protection was given using either nucleic acid in a concentration of 0·06 per cent (w/v) after 15 min contact with peroxide. Thymus nucleic acid gave almost no protection in concentrations up to 0·2 per cent (w/v), about 250 times the concentration of the peroxide on a weight for weight basis. Yeast nucleic acid was more effective, a 0·2 per cent (w/v) solution in contact with 3×10^{-5} M succinyl peroxide reduced the inhibition from 100 to 58 per cent.

The nucleosides, adenosine and guanosine did not protect even when used in a concentration which was 300 times that of peroxide.

Amino acids were examined as possible protective substances in a concentration of 5×10^{-3} M, 150 times that of the peroxide. Only the amino acids cysteine, methionine, histidine and glutathione gave any protection (Table 3).

TABLE 3. PROTECTION OF UREASE BY AMINO ACIDS AGAINST INHIBITION BY SUCCINYL PEROXIDE

(0·005 M amino acid in contact with $3{\cdot}3 \times 10^{-6}$ M succinyl peroxide for 5 min before addition of urease).

Amino acid	Protection (%)	Amino acid	Protection (%)
Glutathione	100	Glycine	0
Cysteine	100	Lysine	0
Methionine	100	Cystine	0
Histidine	60	Asparagine	0
		Leucine	0
		Tryptophan	0
		Threonine	0
		Alanine	0
		Glutamic acid	0
		Tyrosine	0
		Phenylalanine	0
		Valine	0
		Serine	0
		Arginine	0

The effect of histidine was almost completely abolished if the molar ratio of histidine:peroxide was reduced below 25:1 but both cysteine and methionine were extremely effective protectors provided they were used in a concentration which was not less than 3 times that of the peroxide (Table 4).

TABLE 4. THE RELATIVE MOLECULAR RATIO OF CYSTEINE OR METHIONINE:SUCCINYL PEROXIDE WHICH IS REQUIRED TO PROTECT UREASE AGAINST INHIBITION BY SUCCINYL PEROXIDE

(Cysteine or methionine in contact with $3{\cdot}3 \times 10^{-4}$ M succinyl peroxide for 5 min before addition of urease).

	Conc. (M)	Molecular ratio (amino acid: succinyl peroxide)	Protection (%)
Cysteine	$3{\cdot}3 \times 10^{-2}$	10:1	100
	$1{\cdot}6 \times 10^{-3}$	5:1	100
	10^{-3}	3:1	100
	$3{\cdot}3 \times 10^{-4}$	1:1	0
Methionine	$1{\cdot}6 \times 10^{-2}$	5:1	100
	10^{-3}	3:1	100
	$3{\cdot}3 \times 10^{-4}$	1:1	0

Reversibility of succinyl peroxide inhibition of urease

Succinyl peroxide inactivation of urease may be the result of oxidation of —SH groups to —S—S—. If this were the case, complete recovery of activity of the inactivated enzyme should be obtained by the addition of excess of cysteine or glutathione.

In order to investigate the nature of the inhibition, urease was treated with succinyl peroxide in concentrations ranging from 2×10^{-4} M to 2×10^{-5} M for time periods which were varied between 0·25 and 30 min. At the end of the specified time, cysteine was added, to a concentration of 0·005 M. Enzyme activity was then measured and compared with (a) urease treated with the same concentration of peroxide without cysteine and (b) urease with cysteine only added.

Extensive reversal of inhibition was only obtained if either (a) the time of contact of urease and peroxide was very short, or (b) very dilute solutions of peroxide were used. The effect of succinyl peroxide in various concentrations on urease using a 5 min contact period is shown in Table 5. After 30 min contact of peroxide with urease, no reversal could be obtained with any of the peroxide concentrations specified. If the contact time was 15 sec only, 85 per cent recovery of the enzyme activity could be restored when using $3{\cdot}3 \times 10^{-5}$ M succinyl peroxide, but after 25 min no recovery was possible.

Protection of —SH groups of urease against succinyl peroxide

These experiments may be interpreted as indicating that the primary role of succinyl peroxide is likely to be an attack on —SH groups. If this were the case, it would be possible to protect urease against succinyl peroxide by first treating the enzyme with a specific —SH reagent such as *p*-chlormercuribenzoate (*p*-CMB) before adding succinyl peroxide. The *p*-CMB inhibition may then be readily reversed by the addition of excess of cysteine.

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TABLE 5. CYSTEINE REVERSAL OF THE INHIBITION OF UREASE BY SUCCINYL PEROXIDE

(Succinyl peroxide in contact with urease for 5 min before addition of 0·005 M cysteine).

Succinyl peroxide conc. (M)	Cysteine	Inhibition (%)
2×10^{-4}	−	100
2×10^{-4}	+	100
10^{-4}	−	100
10^{-4}	+	90
3.3×10^{-5}	−	100
3.3×10^{-5}	+	66
2×10^{-5}	−	100
2×10^{-5}	+	46

Urease was therefore treated with *p*-CMB (3.3×10^{-4} M) for 3 min, then succinyl peroxide (6.6×10^{-6} M) added and left in contact with the enzyme for 30 min. At the end of this period cysteine was added to give a concentration of 5×10^{-3} M. The cysteine serves a dual purpose; it destroys the succinyl peroxide and reverses the *p*-CMB inhibition by restoring the —SH groups.

A considerable percentage of the total enzyme activity was recovered using the urease that had been treated with *p*-CMB but none at all when using the untreated enzyme (Fig. 2). If the concentration of succinyl peroxide was much greater, e.g.

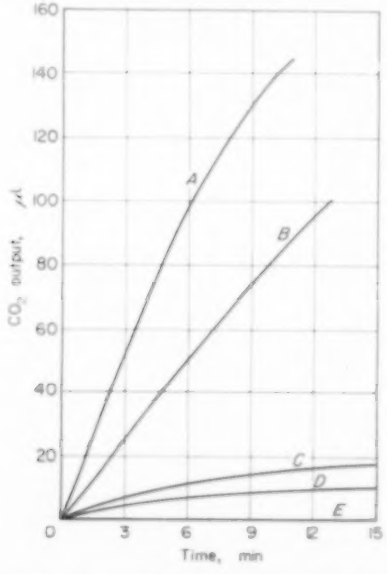

FIG. 2. Effect of succinyl peroxide on urease which has been treated with *p*-chloromercuribenzoate (*p*-CMB). *Curve A*: Control and cysteine only added to *p*-CMB treated enzyme. *Curve B*: Enzyme treated with *p*-CMB before addition of succinyl peroxide, cysteine added at the end of a 30 min period. *Curve C*: Enzyme treated with succinyl peroxide and cysteine added at the end of a 30 min period. *Curve D*: Succinyl peroxide treated enzyme. *Curve E*: *p*-CMB treated enzyme.

5×10^{-5} M, the recovery of enzyme activity was much less and only 20 per cent of the control.

DISCUSSION

Experiments with urease and other —SH enzymes show almost conclusively that succinyl peroxide rapidly attacks sulphydryl groups. The evidence may be summarized in four parts: (a) that —SH enzymes are, in general, much more sensitive to peroxide than are non-SH enzymes; (b) that at least part of the activity of inactivated urease is recovered after the addition of cysteine; (c) that —SH compounds, e.g. cysteine and glutathione, are excellent protectors for enzymes; and (d) that considerable protection against peroxide inhibition may be obtained by blocking the —SH groups.

However, complete recovery of enzyme activity is not possible by cysteine treatment and some inhibition occurs even when —SH groups are blocked. These facts, coupled with observations on the inhibition of some non-SH enzymes, indicate that secondary effects other than simple oxidation of —SH to —S—S— must occur since this type of inactivation is completely and easily reversible.

Certain effects may be termed secondary for several reasons. Firstly, the observation that inhibition of urease by dilute solutions of succinyl peroxide is largely reversible by cysteine but the inhibition by more concentrated solutions is irreversible (Table 5), and secondly, the observation that the inhibition of non-SH enzymes, e.g. invertase, develops more slowly than that of —SH enzymes such as urease (Fig. 1).

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There are several possible secondary effects that may be considered. There may be the irreversible further oxidation of the —S—S— to the sulphoxide or sulphone which is known to occur on treating cystine with hydrogen peroxide[15] or there may be formation of cysteic acid residues. Alternatively, as suggested by the protection experiments with methionine (Table 4) the secondary effect of the peroxide may be a result of the oxidation of a methionine residue to its sulphoxide or sulphone. Methionine has been shown to give a sulphoxide on treatment with hydrogen peroxide in formic acid.[16]

Other possible combining or reaction sites for the peroxide may be either the residues of histidine (the amino acid itself having been found to have a weak protective action); or the ionized carboxyl groups which may be esterified by the free radicals produced by peroxides as suggested by Loveless[3]. However, the fact that inhibition of the enzymes was usually found to increase with increasing acidity implies that combination with carboxyl groups may not be a vital site of combination, since these would tend to become less ionised and therefore less reactive as the pH is made more acid. The interpretation of the effect of pH on the inhibition is, however, complicated by a consideration of the ionization of the persuccinic acid. pK values for this acid are not available but if they may be assumed to be comparable with those of succinic acid (4·19 and 5·57), then changes in the ionization of at least one of the carboxyl groups of the acid will be significant at the pH values used for several of the enzyme tests. It is possible that the undissociated acid is more effective as an enzyme inhibitor than the ion. Different effects observed with different buffers may be accounted for by the combination of the ions of the buffer solution with certain vital sites on the enzyme which would otherwise be liable to peroxide attack.

Proteins were effective as protective agents only if present in a high concentration,

1 mole of protein:2 moles of succinyl peroxide in the case of serum albumin. These results show that there are a few special sites on the protein molecule which are sensitive to peroxide attack. Nucleic acids were much weaker protective substances, as were adenosine and guanosine. A reasonable interpretation of these results is that it is the proteins rather than the nucleic acid which are attacked by peroxides in living organisms.

Many radiomimetic substances are known to have a strong affinity for —SH groups,[17, 18] and it would appear that the radiomimetic properties of succinyl peroxide could be explained as being a result of oxidation of the —SH of cysteine residues. It also appears possible that methionine may be a vital target and it is of interest to note that X-rays have been shown to cause the formation of sulphoxide and sulphone in irradiated methionine solutions.[19]

There are many indications that —SH compounds play a vital role in cell division[20, 21, 22] and furthermore, that sulphydryl groups are specially sensitive to irradiation. Several —SH enzymes have been shown to be strongly inhibited by irradiation[23, 24, 25] and the enzymic activity of solutions of crystalline urease which were used for experiments with succinyl peroxide has been found to be considerably reduced by X-ray doses of 1000 r.[26] However, the greater sensitivity of —SH enzymes to irradiation as compared with that of other enzymes has recently been challenged.[27] The status of the importance of the vulnerability of sulphydryl groups to irradiation is discussed by Bacq and Alexander.[28]

The present work indicates that —SH groups, and the —S of methionine of proteins are very likely targets for peroxide attack, and that if irradiation can give rise to peroxides, as certain experiments have shown, these could well attack —SH compounds, so far unknown, which are vital in the process of cell division.

Acknowledgements—The writer wishes to express his thanks to Professor A. Wormall for advice and interest in this work, to Miss P. Kielty for technical assistance with some of the experiments, and to Dr. R. Latarjet for a gift of succinyl peroxide.

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DEMONSTRATION OF DIRECT CHOLINERGIC RECEPTOR EFFECTS OF METHYLFLUOROPHOSPHORYLCHOLINES

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(*Received* 21 *May* 1959)

Abstract—The effects of four organophosphorylcholines (methylfluorophosphorylcholine, methylfluorophosphoryl-β-methylcholine, methylfluorophosphorylhomocholine and methylfluorophosphorylcarbocholine [3:3-dimethylbutoxyphosphoryl fluoride]) on the frog rectus abdominis muscle and the isolated rabbit duodenum have been studied following complete inhibition of the cholinesterase activity of the organs with *iso*-propoxymethylphosphoryl fluoride (Sarin). All compounds produced contractions of the inhibited rabbit duodenum, but quantitative estimations of the responses were not possible. Such estimations could, however, be performed in experiments on the frog rectus abdominis. The responses are explained in terms of a direct action on cholinergic receptor groups by the compounds.

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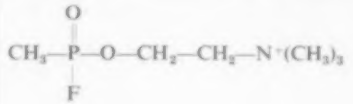
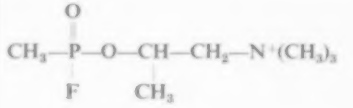
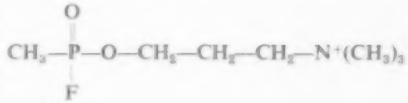
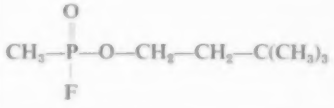
IN two earlier papers,[1, 2] the general pharmacological properties of a series of methylfluorophosphorylcholines (Table 1) were described. These compounds, particularly the three quaternary analogues, are extremely toxic and have a high affinity for cholinesterase, presumably due to their attraction both to the "esteratic" and the "anionic site" of the enzyme.[5, 6] They have structures in common not only with organophosphorus cholinesterase inhibitors but also with the natural substrate of the enzyme, acetylcholine. As the compounds evoked in low doses effects on the cat's blood pressure similar to those produced by choline esters, i.e. a rapid decrease followed by a rapid return to the normal level, it was suggested that the compounds had direct effects on the acetylcholine receptors in addition to their potent indirect activity due to their inhibition of the enzyme. This interpretation was further supported by the fact that the compounds showed the same differences in effect as the corresponding choline esters. Thus, the choline analogue gave both muscarinic and nicotinic responses, the β-methylcholine analogue had only muscarinic effects, the homocholine analogue had both muscarinic and nicotinic effects, and the carbocholine analogue evoked only weak muscarinic responses.

Recently, however, Tammelin[5, 6] has shown that compounds of this type react extremely rapidly with cholinesterase and suggested that the blood pressure effects also might be due to the accumulation of acetylcholine subsequent to the rapid inhibition of the enzyme. This prompted the present writers to see whether the compounds had any effects on isolated organ preparations where the enzyme activity had been completely inhibited in advance with another inhibitor. As test organs the rabbit duodenum was chosen for demonstration of muscarinic effects and the frog rectus for demonstration of nicotinic responses.

TABLE 1

(I) Methylfluorophosphorylcholine	
(II) Methylfluorophosphoryl-β-methylcholine	
(III) Methylfluorophosphorylhomocholine	
(IV) Methylfluorophosphorylcarbocholine	

METHODS

Frog rectus abdominis muscle. The rectus muscle was suspended in a 12 ml bath containing frog-Ringer solution at room temperature. The muscle was incubated for 30 min in 5×10^{-5} M *iso*propoxymethylphosphoryl fluoride (Sarin) in frog-Ringer solution. The muscle was then washed repeatedly, and the preparation was treated with three different concentrations of an acetylcholine standard (Fig. 1). The methylfluorophosphorylcholines were then tested in increasing concentrations until contractions of the same height as those evoked by the acetylcholine standards were reached. Tests with one concentration of the acetylcholine standard was repeated after each test dose. The drugs, which were dissolved in frog-Ringer solution, were allowed to remain in contact with the muscle exactly 90 sec and were then washed out. Five minutes after the wash a new test was performed. The responses were recorded kymographically. Each compound was tested in at least four independent experiments.

Rabbit duodenum. Corresponding experiments were performed in the isolated rabbit duodenum, which was suspended in a 100 ml bath containing Tyrode's solution at a temperature of 34 °C.

Enzyme activity determination. The cholinesterase activity was estimated with the electrometric method described by Tammelin.[7] Acetylcholine iodide of a final concentration of $0{\cdot}73 \times 10^{-2}$ M was used as substrate. The tissues were incubated for 30 min with 5×10^{-5} M Sarin in frog-Ringer solution and then washed repeatedly, also in frog-Ringer solution. The tissues were than homogenized in buffer solution as described by Holmstedt *et al.*,[4] and the enzyme activity determined. The enzyme activity of untreated tissues was examined in control experiments.

RESULTS

The enzyme activity determinations showed that there was complete inhibition of the issue cholinesterases following incubation with 5×10^{-5} M Sarin. This is to be

expected as this concentration of the inhibitor is well over the one known to give complete inhibition of other enzyme sources.[3]

All the methylfluorophosphorylcholines produced contraction of the inhibited rabbit duodenum. Quantitative estimations were, however, not possible as both the test drugs and the acetylcholine standards only evoked minute responses, probably due to the fact that the incubation with Sarin had already produced an intensive contracture of the preparation. Atropinization inhibited the responses completely.

On the frog rectus abdominis muscle, on the other hand, the responses to some of the compounds allowed quantitative comparisons with the acetylcholine standards (Fig. 1). The results are summarized in Table 2 together with effects on the cat's blood

Table 2. Relative cholinergic potency of methylfluorophosphorylcholines as estimated on the frog rectus abdominis muscle and on cat's blood pressure before and after atropine sulphate*

Compound	Number of molecules equivalent to one molecule of acetylcholine		
	Frog rectus	Cat blood pressure*	
		Depressor response	Pressor response (after atropine)
Acetylcholine	1	1	1
(I) Methylfluorophosphorylcholine	18	90	20
(II) Methylfluorophosphoryl-β-methylcholine	>4000	110	—
(III) Methylfluorophosphorylhomocholine	550	150	60
(IV) Methylfluorophosphorylcarbocholine	>4000	400	—

*From Fredriksson[2]

pressure before and after atropinisation.[2] There were no cumulative effects and repeated doses gave no decrease in response. The initial response to the acetylcholine standard was unaltered throughout the experiments (Fig. 1).

DISCUSSION

The immediate cholinergic responses of the compounds in the cat, as evidenced by rapid blood pressure changes before and after atropine, were for two reasons assumed to be dependent on direct effects on receptors and not merely on a liberation of cholinergic transmittors or their accumulation following inhibition of cholinesterase.[2] Thus, the compounds had different effects, and these effects resembled those produced by the corresponding choline esters. This theory seems to be supported by the present experiments. That the compounds evoked responses in spite of complete inhibition of cholinesterase, produced by a compound known to give very slowly reversible inhibition, shows that the immediate effects are due to direct and not indirect cholinergic acitivity. Not only the enzyme activity determinations but also the fact that the response to the acetylcholine standard was unaltered throughout the experiments show that there was complete inhibition of the enzyme activity. Furthermore, the responses can hardly be explained by release of cholinergic transmittors from the tissues since repeated doses of the test substances always gave the same response. Thus, there was

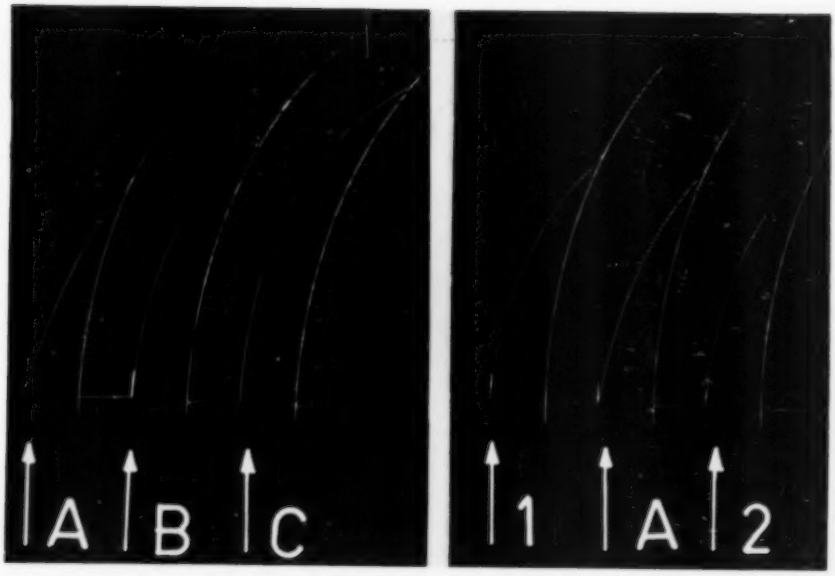


FIG. 1. Effects of methylfluorophosphorylcholine on the Sarin-treated frog rectus abdominis muscle as compared with acetylcholine. At A, B and C acetylcholine (0·09, 0·18 and 0·27 μg ml bath fluid, respectively). At 1 and 2 methylfluorophosphorylcholine (1·5 and 2·25 μg ml bath fluid, respectively.)

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no decrease in the response even after more than ten repeated doses of the compounds. The immediate effects of the substances are thus due to cholinergic receptor activity, which is reasonable in view of their structural similarity with cholinergic transmittors.

The compounds have different relative potencies in the cat's blood pressure and the frog rectus preparations. Such species differences are to be expected. Furthermore, the two types of experiments are not directly comparable: in the frog rectus there was complete inhibition of the enzyme activity, whereas this certainly was not the case in the cat.

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THE PHARMACOLOGICAL EVALUATION OF MONOAMINE OXIDASE INHIBITORS

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(*Received* 4 *June* 1959)

Abstract—It is shown that the prevention of reserpine hypothermia by various compounds is related to their ability to inhibit brain monoamine oxidase activity. The tremors produced by DL-5-hydroxytryptophan in mice pretreated with an amine oxidase inhibitor have been measured. The intensity of the tremors depend upon the degree of monoamine oxidase inhibition and the dose of the tremoring agent. The administration of harmaline, a potent and reversible inhibitor of monoamine oxidase activity can block the potentiation of DL-5-hydroxytryptophan by irreversible inhibitors.

IPRONIAZID (*iso*propyl*iso*nicotinic acid hydrazide) is a potent *in vitro* and *in vivo* inhibitor of monoamine oxidase.[1, 2] The single administration of this compound to rabbits or mice does not produce any marked alteration in behaviour or activity, but when followed by reserpine a marked excitation may be observed.[3, 4, 5]

In the mouse, reserpine produces sedation and the degree of sedation produced may conveniently be followed by measuring the fall in rectal temperature.[6] After a suitable dose of iproniazid reserpine no longer produces sedation or hypothermia.[7, 8] The degree of antagonism by iproniazid is related to the extent to which the brain monoamine oxidase has been inhibited.[8]

Other monoamine oxidase inhibitors have been examined by this method in an attempt to correlate brain monoamine oxidase inhibition with reserpine antagonism.

The administration of DL-5-hydroxytryptophan to animals elicits symptoms of postural inco-ordination, loss of placing reactions and generalized skeletal muscle tremors. In mice and rats these effects can only be obtained in animals pretreated with iproniazid.[9, 10] Our preliminary experiments confirmed this, and a method was devised to measure the tremors produced by DL-5-hydroxytryptophan in mice treated with various inhibitors of monoamine oxidase.

METHODS

LAC grey mice (18–22 g body weight) of either sex were used. The drugs were administered intraperitoneally (0·1 ml/10 g body weight). The reserpine was dissolved in 2 N acetic acid, diluted and neutralized just before use. The room temperature was maintained as far as possible between 20–22°C.

Reserpine antagonism. Groups of mice were treated with reserpine (2 mg/kg i.p.) and the rectal temperatures determined 4 hr later. This dose of reserpine produced a temperature fall of about 5–7 °C in this time.

Measurement of tremor. Mice were placed singly in a thin aluminium tube 7 cm long and 3 cm in diameter. The tube was suspended horizontally on a gramophone pick-up

cartridge (Acos G.P. 13-6). The vibrations of the tube were fed to an amplifier. The output from the cathode follower stage was connected to a 24 V low inertia integrating motor and counter (Electro Methods Ltd.). Any increase or decrease in the frequency or amplitude of tremors was reflected in changes in the speed of the motor and counter.

The tremors were produced by injecting a suitable dose of DL-5-hydroxytryptophan i.p. into groups of mice. The mean 2 min count was determined 30–50 min after the administration of the DL-5-hydroxytryptophan.

RESULTS

Reserpine antagonism. It has already been shown that the complete inhibition of brain monoamine oxidase by iproniazid takes several hours. For this reason iproniazid was given 4–6 hr before the reserpine. The effect of varying the dose of iproniazid upon the hypothermia produced by reserpine is shown in Fig. 1. This shows that whereas 25 mg/kg iproniazid had no protecting action against the reserpine hypothermia, 100 mg/kg was able to give complete protection.

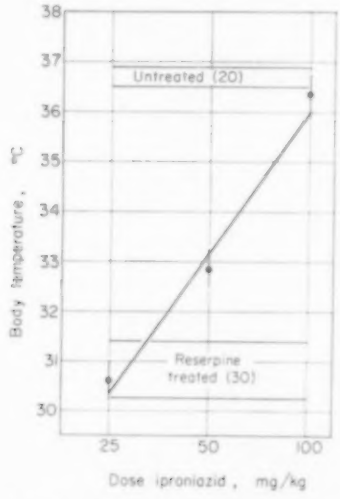

FIG. 1. The effect of various doses of iproniazid upon reserpine hypothermia. No. of values = 60; correlation = 0·83; slope = 9·4 ± 1·3 (95% limits).

Other hydrazides were examined by this method. These agents were given 4–6 hr before the reserpine. In addition harmaline was used and injected into the mice 1 hr before the reserpine. These time intervals were chosen after consideration of the time-course of action of these drugs by other techniques. The results have been tabluated and are shown in Table 1. The hydrazides reduced the activity of brain monoamine oxidase by 50 per cent when the animals were treated with the doses listed in the last column. These estimations were kindly carried out by Dr. R. F. Long* using a method similar to that described by Davison and Sandler.[11] It may be noted that these compounds were more active than iproniazid, both as reserpine antagonists and monoamine oxidase inhibitors.

*Of Roche Products Ltd.

TABLE 1. THE ACTIVITY OF VARIOUS HYDRAZIDES AND HARMALINE AS RESERPINE ANTAGONISTS

Compound	Dose (mg/kg)	Time before reserpine	Body temp. (°C) (A) Both drugs given	(B) Untreated	(C) Reserpine alone	Difference (B–A)	Difference (A–C)	Dose (mg/kg) for 50% inhibition
*iso*propyl *iso*nicotinic acid hydrazide iproniazid	25	4 hr	30·4 ± 0·4 (20)	36·5 ± 0·2 (10)	30·25 ± 0·3 (40)	$P < 0.001$	$P > 0.7$	20
	100	4 hr	36·1 ± 0·3 (10)	36·5 ± 0·2 (10)	30·25 ± 0·3 (40)	$P > 0.3$	$P < 0.001$	
Harmaline	10	15 min	32·8 ± 0·4 (10)	35·5 ± 0·3 (10)	29·4 ± 0·3 (10)	$P < 0.001$	$P < 0.001$	—
	20	15 min	35·3 ± 0·3 (10)	35·5 ± 0·3 (10)	29·4 ± 0·3 (10)	$P = > 0.6$	$P < 0.001$	
L-Glutamic acid α-(*iso*propyl-hydrazide) Ro 4-1385	5	4 hr	35·3 ± 0·6 (8)	36·0 ± 0·4 (8)	31·4 ± 0·4 (8)	$P > 0.3$	$P < 0.001$	4
	10	4 hr	35·7 ± 0·5 (5)	36·2 ± 0·8 (5)	32·8 ± 0·5 (5)	$P \simeq 0.6$	$P < 0.01$	
DL-Serine-N²-*iso*propyl hydrazide hydrochloride, Ro 4-1038/1	5	4 hr	34·5 ± 0·7 (8)	36·0 ± 0·4 (8)	31·4 ± 0·4 (8)	$P = 0.05 - 0.1$	$P < 0.01$	5
	10	4 hr	35·1 ± 0·2 (5)	36·2 ± 0·8 (5)	32·8 ± 0·6 (5)	$P > 0.2$	$P < 0.01$	
L-α-Alanine-*iso*propyl hydrazide monohydrochloride Ro 4-1340	5	4 hr	35·0 ± 0·8 (8)	36·0 ± 0·4 (8)	31·4 ± 0·4 (8)	$P > 0.2$	$P < 0.001$	3
	10	4 hr	35·8 ± 0·3 (5)	36·2 ± 0·8 (5)	32·8 ± 0·6 (5)	$P > 0.6$	$P < 0.01$	

The compounds were administered at intervals before the reserpine (2·0 mg/kg i.p.), the temperatures measured 4 hr later. The mean temperature of the treated animals was compared with the means of untreated animals and animals receiving reserpine. In the last column the dose of each inhibitor producing a 50 per cent reduction in monoamine oxidase activity in the homogenized brains of animals treated with the compounds is shown. No. of values used shown in brackets.

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Production of tremors. Although iproniazid or DL-5-hydroxytryptophan do not produce tremors in mice in doses up to 200 mg/kg, when the DL-5-hydroxytryptophan (50 mg/kg) is administered 4 hr after the iproniazid (200 mg/kg) tremors may be observed. The tremors are apparent about 10 min after injection and are maximal at 30–50 min (Fig. 2).

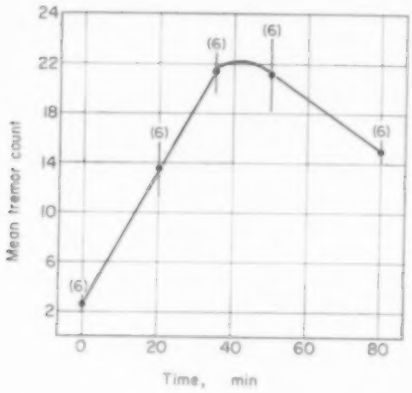

FIG. 2. The time course of the tremors produced by 5-hydroxytryptophan (50 mg/kg i.p.) in animals pretreated with iproniazid (200 mg/kg i.p.). Number of values shown in brackets.

The optimal interval between the iproniazid and the DL-5-hydroxytryptophan was established as 4–6 hr in a series of experiments where the interval between the two agents was varied (Fig. 3).

By injecting doses of iproniazid 4–6 hr before the DL-5-hydroxytryptophan (100

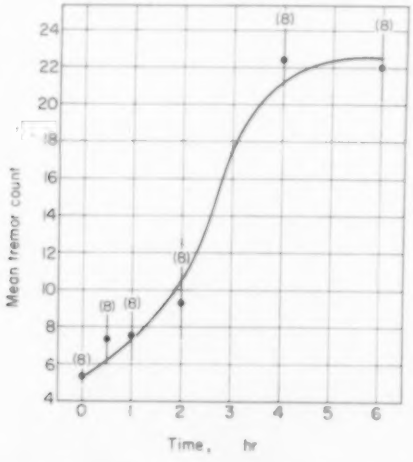

FIG. 3. The production of tremors by 5-hydroxytryptophan (100 mg/kg i.p.) administered at various intervals (abscissa) after iproniazid (100 mg/kg i.p.). Number of values shown in brackets. The readings were taken 30–50 min after the injection of the 5-hydroxytryptophan.

mg/kg) it was possible to construct a dose-response curve for this hydrazide (Fig. 4). The correlation between dose and effect was highly significant ($r = 0{\cdot}89$) when doses of iproniazid between 25 and 200 mg/kg were used. With doses above 200 mg/kg there was no further increase in the mean tremor count, whereas with doses below 25 mg/kg no consistent tremoring effect could be obtained.

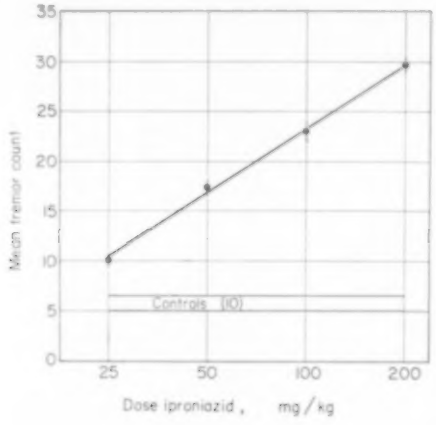

FIG. 4. The effect of 5-hydroxytryptophan (100 mg/kg i.p.) given 4–6 hr after various doses of iproniazid. No. of values = 36; correlation = 0·89; slope = 20·8 ± 3·7 (95% limits).

It was also found possible to construct a dose-response curve for the DL-5-hydroxytryptophan by pretreating the animals with a fixed dose of iproniazid (200 mg/kg) 4–6 hr before various doses of the tremoring agent. In Fig. 5 the curve obtained is shown using doses of DL-5-hydroxytryptophan between 25 and 100 mg/kg. Doses greater than 100 mg/kg produced no further increase in the count, whilst doses below 25 mg/kg were ineffective in producing tremors.

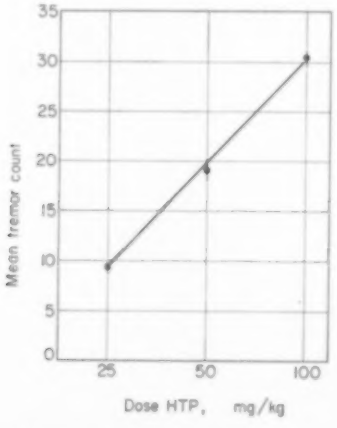

FIG. 5. Effect of various doses of 5-hydroxytryptophan given 4–6 hr after iproniazid (200 mg/kg i.p.) No. of values = 41; correlation = 0·86; slope = 34·9 ± 6·4 (95% limits).

The two isomers of 5-hydroxytrytophan were compared for their ability to produce tremors, and Table 2 shows that the D-form was inactive in doses up to 100 mg/kg. The L-form had the greatest activity, about twice that of the DL-racemate.

TABLE 2. THE ACTIVITY OF THE OPTICAL ISOMERS OF 5-HYDROXYTRYPTOPHAN IN PRODUCING TREMORS

Compound	25 mg/kg	50 mg/kg	100 mg/kg
D-5-Hydroxytryptophan	—	—	2·0 ± 1·0 (6)
L-5-Hydroxytryptophan	23·1 ± 2·1 (6)	33·2 ± 3·3 (6)	—
DL-5-Hydroxytryptophan	—	25·7 ± 0·5 (6)	33·3 ± 1·8 (6)

Mice treated 4–6 hr earlier with iproniazid (200 mg/kg). No. of values is given in brackets.

The three hydrazides examined earlier as reserpine antagonists were now tested for their ability to potentiate DL-5-hydroxytryptophan tremors. The optimal interval between injection of each hydrazide and the tremoring agent was determined. Table 3 shows that these compounds exert their effect much more rapidly than iproniazid.

TABLE 3. THE EFFECT OF VARYING THE INTERVAL BETWEEN THE ADMINISTRATION OF VARIOUS HYDRAZIDES UPON THE TREMORS PRODUCED BY 5-HYDROXYTRYPTOPHAN

Compound	Dose (mg/kg)	Interval between drug and 5-hydroxytryptophan (100 mg/kg)				
		0 min	15 min	30 min	1 hr	1½ hr
L-Glutamic acid-α-*iso*propylhydrazide Ro 4–1385	32	10·4 ± 0·9 (5)	11·8 ± 0·9 (5)	14·2 ± 2·5 (5)	20·4 ± 2·6 (5)	31·4 ± 1·2 (5)
L-α-Alanine *iso*-propylhydrazide monohydrochloride (Ro 4–1340)	24	23·2 ± 1·2 (5)	27·2 ± 1·6 (5)	34·0 ± 1·6 (5)	30·3 ± 1·5 (5)	—
DL-Serine-N²-*iso*-propyl hydrazide monohydrochloride (Ro 4–1038/1)	24	13·4 ± 3·0 (5)	17·4 ± 2·4 (5)	20·2 ± 1·7 (5)	30·4 ± 2·5 (5)	—

No. of values is given in brackets. The readings were taken 30–50 min after the injection of the 5-hydroxytryptophan.

All exert some effect when given simultaneously with the tremoring agent; indeed the L-α-alanine *iso*propyl hydrazide was almost fully effective under these conditions. Dose response curves for these compounds were constructed (Fig. 6) by injecting various doses 2–4 hr before the DL-5-hydroxytryptophan (100 mg/kg). All three hydrazides were found to be from five to ten times as active as iproniazid.

Harmaline is known to be a reversible inhibitor of monoamine oxidase. The reversible nature of this compound is illustrated in Fig. 7 which shows that 8 hr after a dose of 5 mg/kg no tremoring effect with DL-5-hydroxytryptophan (100 mg/kg) could be observed. These results agree with those of Udenfriend and his co-workers,[12] who

showed a similar time course for the inhibition of monoamine oxidase by this drug. Harmaline in higher doses produces intention tremors but at the dose level used these did not register on the counter.

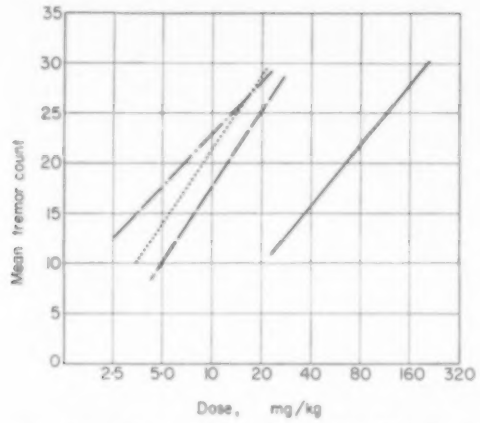

FIG. 6. Dose-response curves for various hydrazide derivatives The dose of 5-hydroxytryptophan (100 mg/kg) was administered 4–6 hr after the hydrazides.

——— Iproniazid: no. of values = 36; correlation = 0·89; slope = 20·8 ± 3·7 (95% limits).

— — L-Glutamic acid-α-*iso*propyl hydrazide (Ro 4–1385): no. of values = 38; correlation = 0·87; slope = 24·9 ± 4·6 (95% limits).

— · — · L-α-Alanine-*iso*propyl hydrazide hydrochloride (Ro 4–1340): no. of values = 32; correlation = 0·85; slope = 18·5 ± 4·1 (95% limits).

· · · · · · DL-serine-N^2-*iso*propyl hydrazide hydrochloride (Ro 4–1038/1): no. of values = 39; correlation = 0·89; slope = 25·6 ± 4·2 (95% limits).

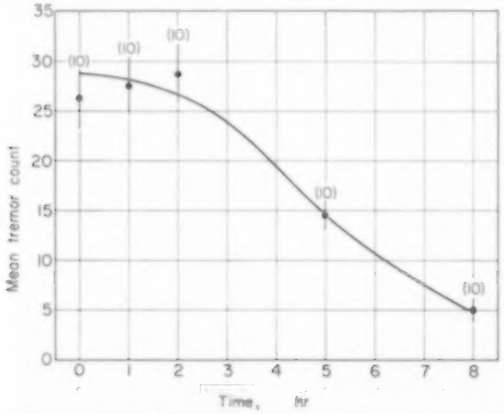

FIG. 7. The time-course for the effect of harmaline in potentiating DL-5-hydroxytryptophan tremors. The harmaline (5 mg/kg i.p.) was administered at various intervals before the DL-5-hydroxytryptophan (100 mg/kg i.p.).

When harmaline was administered to animals 15 min before an irreversible inhibitor such as L-α-alanine *iso*propyl hydrazide and DL-5-hydroxytryptophan injected 7 hr later, no tremors were observed, that is the effect of the hydrazide was prevented (Table 4).

TABLE 4. TABLE ILLUSTRATING THE EFFECTIVENESS OF HARMALINE IN PREVENTING THE TREMORS NORMALLY OBSERVED WHEN 5-HYDROXYTRYPTOPHAN IS GIVEN TO ANIMALS TREATED WITH L-α-ALANINE-*iso*PROPYL HYDRAZIDE MONOHYDROCHLORIDE

Treatment	Tremor count
Animals given harmaline (5 mg/kg) and 15 min later 5-hydroxytryptophan (100 mg/kg)	28·9 ± 2·0 (10)
Animals given harmaline (5 mg/kg) and 7 hr later 5-hydroxytryptophan (100 mg/kg)	4·3 ± 0·9 (10)
Animals given L-α-alanine-*iso*propyl hydrazide monohydrochloride (20 mg/kg) and 7 hr later 5-hydroxytryptophan (100 mg/kg) (Ro 4-1340)	30·0 ± 3·5 (10)
Animals given harmaline (5 mg/kg) and 15 min later L-α-alanine *iso*propyl-hydrazide (20 mg/kg) and 7 hr later 5-hydroxytryptophan (100 mg/kg)	4·8 ± 1·0 (10)

DISCUSSION

The ability of iproniazid to prevent reserpine hypothermia has already been established,[7] and this has now been shown to be a property of other hydrazides and also of harmaline. Furthermore, there is a reasonable correlation between the activities of these hydrazides as reserpine antagonists and brain monoamine oxidase inhibitors. It is reasonable to conclude that the prevention of reserpine hypothermia is a fundamental consequence of brain monoamine oxidase inhibition particularly since harmaline, an inhibitor of a different structure also shares this property.

However, the delay of 4 hr between the administration of reserpine and the measurement of the hypothermia can give rise to misleading results when using inhibitors such as harmaline which have such a transient effect on the enzyme. The other method described avoids this difficulty as the measurements of the tremors are made 30–50 min after injection of the DL-5-hydroxytryptophan. That this method gives comparable results is clear from the experiments illustrated, the hydrazides being equally active in both tests.

When DL-5-hydroxytryptophan is injected into animals, the L-form is decarboxylated in the brain giving 5-hydroxytryptamine.[13, 14] This agrees with the view that the tremors are due to an accumulation of brain 5-hydroxytryptamine since, as shown earlier the D-5-hydroxytryptophan was devoid of activity. Furthermore, the effectiveness of DL-5-hydroxytryptophan in producing tremors is greatest when the monoamine oxidase is fully inhibited, i.e. 4 hr after iproniazid or 0–2 hr after harmaline. This would lead to the greatest protection of the 5-hydroxytryptamine formed by preventing its degradation to 5-hydroxyindole acetic acid.

The prevention of the action of L-α-alanine *iso*propylhydrazide by harmaline in the tremor test is further evidence that the tremors obtained with DL-5-hydroxytryptophan depend upon the brain monoamine oxidase activity since it has been shown[15] that the irreversible inhibition of brain monoamine oxidase activity by iproniazid and similar compounds is prevented when animals are pretreated with harmaline.

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INHIBITION OF *STREPTOCOCCUS PYOGENES* BY 1:2-DIHYDRO-*s*-TRIAZINES AND SULPHONAMIDES, *in vitro*; MECHANISM OF REVERSAL

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Abstract—A chemically defined culture medium for *Streptococcus pyogenes* C–203 is described. The addition of DNA* and acetate, but not of pteroylglutamic acid or leucovorin, to this medium reverses the inhibitory effect of 1:2-dihydro-*s*-triazines on this organism. RNA supplemented with acetate is inactive in this reversal but a combination of deoxyguanylic acid and thymidine is nearly as active as DNA. 1:2-Dihydro-*s*-triazines are synergistic with sulphonamides against *S. pyogenes* in the defined medium but not in crude media. DNA or its constituent deoxyribosides reverse this synergism but PABA is ineffective. *S. pyogenes* remains sensitive to 1:2-dihydro-*s*-triazines after repeated transfers in a defined culture medium containing no thiamine, and develops very little tolerance to these compounds following the addition of acetate and guanine to the medium; but the further addition of thiamine causes a greatly increased tolerance. This increase in tolerance of *S. pyogenes* to 1:2-dihydro-*s*-triazines, which develops with repeated transfers and is caused by the addition of acetate, guanine, and thiamine to the defined medium, is blocked by sulphonamides.

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INTRODUCTION

IN an accompanying communication,[1] Fisher and Doub have described the inhibitory activities of a representative 1:2-dihydro-*s*-triazine against a number of bacterial pathogens, both *in vitro* and *in vivo*, and also have referred to previously published chemical and microbiological work by others on the 1:2-dihydro-*s*-triazines. These workers[1] observed synergism between the 1:2-dihydro-*s*-triazines and sulphonamides in mice but were unable to demonstrate such action in their studies *in vitro*, using a crude medium. We wish to report the development of a chemically defined culture medium for *Streptococcus pyogenes* C-203 and, further, certain observations concerning the mechanism of the inhibitory action of the 1:2-dihydro-*s*-triazines, acting alone and synergistically with the sulphonamides, against this micro-organism.

MATERIALS AND METHODS

Stock cultures of *Streptococcus pyogenes* C-203 were maintained by monthly stab inoculation into TSB medium containing 1% agar, incubation of these cultures for 24 hr at 37 °C, and storing them in a refrigerator. Inoculum was prepared by centrifuging a fresh 16 hr culture, grown in liquid TSB medium, and resuspending the packed organisms in sufficient 0·85% solution of sodium chloride to give a transmission reading of 30 on a model 400 Lumetron photoelectric colorimeter with a

*The following abbreviations are used: PGA, pteroylglutamic acid; CF, citrovorum factor; PABA, *p*-aminobenzoic acid; TSB, trypticase soy broth; DNA, deoxyribose nucleic acid; RNA, ribose nucleic acid; CoA, coenzyme *A*.

green filter. Two millilitres of this suspension of organisms was then added to 100 ml of double strength medium which previously had been prepared and sterilized as indicated in Table 1.

TABLE 1. CHEMICALLY DEFINED MEDIUM FOR *Streptococcus pyogenes* C-203

	Dissolve in 300 ml H_2O:
Part I	
Amino acid mixture*	8 g
Asparagine	400 mg
Cysteine·HCl	250 mg
KH_2PO_4	6 g
Na_2HPO_4	2 g
Salt mixture†	4 ml
Pyridoxal·HCl	2 mg
Nicotinic acid	2 mg
Riboflavin	2 mg
Calcium pantothenate	2 mg
Thiamine·HCl	2 mg
Biotin	20 μg
Adjust to pH 6·2, dilute to 400 ml and autoclave 10 min at 120 °C.	
Part II	
Dissolve 30 g $NaHCO_3$ in 500 ml H_2O. Add: 0·1 ml thioglycollic acid, 50 mg glutathione, 500 mg glutamine. Filter through Seitz filter.	
Part III	
Dissolve in water and dilute to 100 ml: Glucose 40 g; $CaCl_2·2\ H_2O$ 50 mg. Autoclave 10 min at 120 °C Combine aseptically parts I, II and III and dilute to 1 l. to make double-strength medium. Final pH should be 7·2–7·5.	

*Mix and grind to a powder 1 g each of DL-alanine, L-arginine, DL-aspartic acid, L-cystine, L-glutamic acid, L-histidine, DL-lysine, DL-methionine, DL-phenylalanine, DL-serine, DL-threonine, DL-valine, L-tyrosine, DL*iso*leucine, L-proline, glycine and 2 g each of DL-leucine and DL-tryptophan. Dissolve the required amount by adding a little HCl and boiling.

†25 g $MgSO_4·7\ H_2O$; 20 mg $MnCl_2·4\ H_2O$; 2 mg $FeSO_4·7\ H_2O$; 2 mg $ZnSO_4·7\ H_2O$; 5 mg $CuSO_4·5\ H_2O$; and water to 200 ml.

Note: Part I can be stored frozen. Parts II and III should be made fresh each time.

Experiments were set up by adding predetermined amounts of distilled water and all additives, other than 1:2-dihydro-*s*-triazines (hereafter referred to as triazines) and sulphonamides, to 16 × 150 mm test tubes and sterilizing them by heating for 10 min at 120 °C. Triazines and sulphonamides were sterilized separately by dissolving them in 70% ethanol, diluting with sterile distilled water, and adding the proper amounts of these dilutions aseptically to the previously sterilized and cooled tubes, making a final volume of 1·25 ml of liquid in each tube. To each of these tubes was added aseptically 1·25 ml of inoculated medium, then all were placed in a large container and covered tightly with a thin rubber sheet having a tube sealed into it through which gas could be passed. The covered container was then flushed with CO_2 by alternately forcing CO_2 into it from a tank and then evacuating, finally leaving the CO_2 under a slight pressure in the covered container. The container was then placed in an incubator and maintained at 37 °C for 24 hr, or other stated incubation time. No more CO_2

was added during this incubation since it was apparently important only in initiating growth uniformly. Turbidity readings of all tubes were then made in the colorimeter and recorded as percentage transmission.

The triazine used throughout the bulk of the work reported here was 1-(3′-chlorophenyl)-2:2-dimethyl-4:6-diamino-1:2-dihydro-*s*-triazine. Two other triazines, which are referred to as triazine 2 and triazine 3, were used in supplementary experiments. Triazine 2 is identical with the above-named triazine except for the substitution of a 3:5-dichlorophenyl group for the 3-chlorophenyl group; triazine 3 is also the same except for a 3:4-dichlorophenyl group in place of the 3-chlorophenyl group and only one CH_3 group in the 2-position. Triazine 3 is the compound used in our previous study[2] of the mechanism of inhibitory action of folic acid antagonists in non-folic acid requiring organisms. The source of these triazines has been indicated.[1] The sulphonamide used for most of these studies, and indicated simply as sulphonamide in text and tables, was a mixture containing equal parts sulphadiazine, sulphamethazine, and sulphamerazine. Also used in supplementary experiments were sulphanilamide and sulphamethoxypyridazine (Midicel*).

EXPERIMENTAL RESULTS

Development of defined media

Preliminary phases of the work reported here confirmed the observations of Fisher and Doub[1] that triazines are effective inhibitors of *S. pyogenes* in TSB medium and that triazines and sulphonamides are not synergistic in this medium. A defined medium was needed with which to investigate these relationships and the lack of synergism in TSB medium.

A defined medium for the growth of *S. pyogenes* has been described by Pappenheimer.[3] This utilized acid-hydrolysed gelatin as an amino acid source and also contained supplemental amino acids, purines, vitamins and trace minerals. Incubation of cultures was carried out in an atmosphere containing added CO_2. Although our culture of *S. pyogenes*, possibly due to strain characteristics, would not grow in this medium, it served as a basis for further development. Of various modifications of Pappenheimer's medium tried, the most successful was one in which 20 g of casamino acids,† supplemented with 50 ml of a 5% solution of casein hydrolysate (enzymic)‡ was used in place of the amino acid mixture and asparagine in the final medium described in Table 1. This medium, referred to as the enzymed casein–casamino acids medium, was used in many of the intermediate studies described in this paper. It was found that substituting the mixture of amino acids shown in Table 1 for casamino acids gave good growth of the organism provided the enzymed casein component was retained. Finally, it was found that asparagine could substitute for the enzymed casein fraction, giving the complete growth medium shown in Table 1. The growth of *S. pyogenes* on this medium, although somewhat deficient after only 16 hr incubation, was equal to that on crude medium at 20 hr and exceeded it after longer incubation. This medium was the one used in the latter definitive studies on the reversal of triazines and the synergism between triazines and sulphonamides reported in this paper.

*The trade name of Parke, Davis and Company for 3-sulphanilamido-6-methoxypyridazine.
†Difco Company.
‡Nutritional Biochemicals Corporation.

Reversal factors for triazines

The fact that more triazine was required to give half inhibition of *S. pyogenes* in TSB medium than in enzymed casein–casamino acids medium indicated that the former contained a substance causing reversal of the triazine. Conditions were set up, using the latter medium, to assay natural substances for their content of this triazine-reversing substance. Table 2 shows the results of assaying several of the most active

TABLE 2. DEMONSTRATION OF TRIAZINE-REVERSING FACTOR IN NATURAL SUBSTANCES USING ENZYMED CASEIN–CASAMINO ACIDS MEDIUM CONTAINING 0·06 μg TRIAZINE PER ml

Substance added	Required for complete reversal
	(mg/ml)
Trypticase soy solids*	40
Yeast extract†	2
Ribose nucleic acid‡	2
Deoxyribose nucleic acid§	0·05

*Baltimore Biological Laboratories.
†Difco.
‡Yeast nucleic acid (California Corporation for Biochemical Research).
§Sperm nucleic acid (California Corporation for Biochemical Research).

Growth controls had a Lumerton transmission reading of 25 (20 hr incubation). Completely inhibited culture read 100; completely reversed culture read 25.

natural sources. Sperm nucleic acid (DNA) was by far the most potent substance found. Note that yeast nucleic acid (RNA) was no more potent than yeast extract, probably indicating that the observed activity of yeast nucleic acid was due to the presence of a small amount of DNA. Pure substances which were *inactive* in reversing the inhibitory effect of triazine under these conditions included PGA, leucovorin, PABA, ascorbic acid, nicotinic acid and glutathione, alone or in combination.

Although DNA was quite active in reversing the inhibitory action of triazine on *S. pyogenes* when added to the enzymed casein–casamino acids medium, DNA which had been hydrolysed by heating at 120 °C for 30 min in 1 N HCl, which would presumably free the purine and pyrimidine bases, was inactive. Heating DNA 30 min at 120 °C in 1 N NaOH caused no loss of activity. A milder acid treatment of DNA, heating at 100 °C at pH 1·6 according to the method of Tamm *et al.*[4], resulted in a product which was still active but which lost its activity on dialysis. This dialysed product has been referred to as "apurinic acid", since it presumably lacks the purine bases associated with DNA. Supplementing apurinic acid with various purine bases restored the bulk of the original activity of DNA. In Table 3 are indicated degrees of reversal of triazine obtained, after two incubation periods, with DNA, various pure compounds representing hydrolytic products of DNA alone and in combination, and with RNA for comparison. Next to DNA, a mixture of deoxyguanylic acid and thymidine was most active, a mixture of guanine and thymidine being essentially as active. RNA had very low activity.

In experiments to elucidate the function of the remaining undefined supplement in the enzymed casein–casamino acids medium which was necessary for reversing the inhibitory effect of triazines, namely enzymed casein, a basal medium was used which

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TABLE 3. RELATIVE ACTIVITIES OF RNA AND DNA AND ITS COMPONENTS IN CAUSING REVERSAL OF TRIAZINE IN ENZYMED CASEIN–CASAMINO ACIDS MEDIUM

Substance added per ml	Inhibition time (hr)	
	20	40
	(Triazine required for half inhibition) (μg/ml)	
None	0·03	0·06
100 μg RNA	0·06	0·3
100 μg DNA	2·0	5·0
20 μg deoxyguanylic acid	0·1	0·5
20 μg each of deoxyguanylic acid and thymidine	0·15	5·0
20 μg thymidine	0·03	0·1
20 μg guanine	0·06	0·2
20 μg each of guanine and thymidine	0·1	5·0
20 μg each of adenylic acid and thymidine	0·01	0·5
20 μg adenylic acid	0·01	0·1
20 μg deoxycytodylic acid	0·03	0·1
20 μg each of deoxyguanylic acid, deoxyadenylic acid, and thymidine and 10 μg deoxycytosine	0·01	4·0

contained casamino acids and was supplemented with 200 μg of DNA per ml and from which enzymed casein had been omitted. As indicated in Table 4, when each millilitre of this medium was supplemented with 0·02 ml of the commercial preparation of enzymed casein there was a fiftyfold increase in the amount of triazine necessary for half inhibition of *S. pyogenes*. However, a casein hydrolysate prepared in our laboratory by incubating casein with fresh beef pancreas and neutralizing the resulting mixture with HCl contributed nothing to the triazine-reversing effect of the medium to which it was added. Suspecting that the commercial enzymed casein might contain acetate, since it was probably based on a preparation originally suggested by Roberts and Snell[5], sodium acetate was then added to the medium and found to be equally as effective in reversing the triazine as had been the commercial preparation of enzymed casein. Furthermore, the relationship between acetate added and the resulting amount of triazine required for inhibition was competitive, indicating that the triazine might be blocking the utilization of acetate. As confirmation that the effect of the commercial enzymed casein in reversing the inhibitory effect of triazine had been due to its acetate

TABLE 4. REVERSAL OF TRIAZINE BY THE COMPONENTS OF ENZYMED CASEIN IN MEDIUM CONTAINING 200 μg DNA PER ml

Substance added per ml	Triazine required for half-inhibition* (μg/ml)
None	0·02
0·02 ml commercial enzymed casein†	1·0
0·1 ml laboratory prepared enzymed casein‡	0·02
1 mg sodium acetate	1·0
2 mg sodium acetate	2·0

*20-hr incubation.
†5% solution of enzymic hydrolysate of casein (Nutritional Biochemicals Corporation).
‡5% casein incubated with beef pancreas and neutralized with HCl.

content, a microbiological assay with *Lactobacillus acidophilus* ATCC no. 4963, based on the method suggested by Roberts and Snell[5], and using a medium from which acetate had been omitted, indicated the presence of 0·8 mg of acetate in 0·02 ml of the enzymed casein preparation originally used in the medium.

Synergism of triazines and sulphonamides

Since Fisher and Doub[1] had been unable to demonstrate synergism of triazines and sulphonamides against *S. pyogenes* in TSB medium, we were interested in finding whether synergism occurred between these two types of compounds in the partially defined enzymed casein–casamino acids medium and in the completely defined amino acid medium (Table 1). Results of tests with these two media, as well as TSB medium, are shown in Fig. 1, in which the data are plotted according to the procedure devised

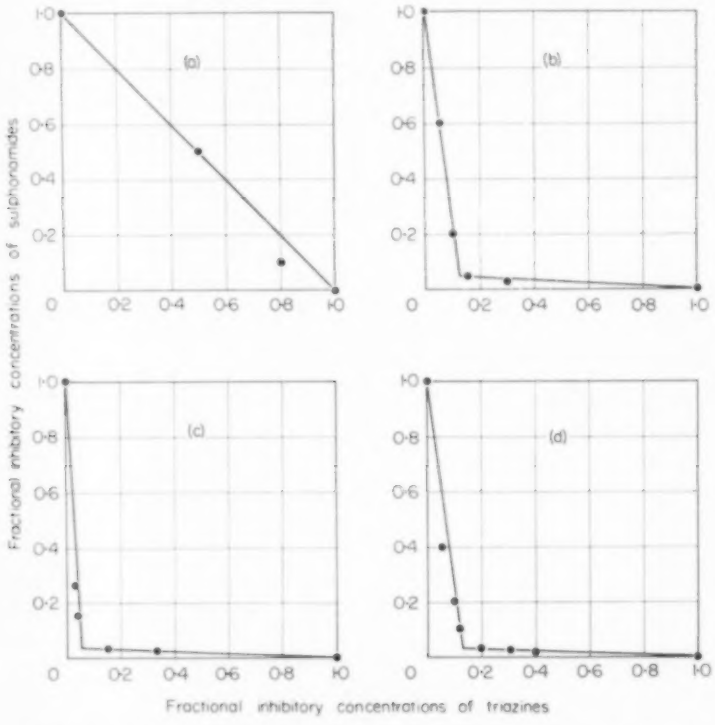

FIG. 1. Plots showing effects of combinations of sulphonamides and dihydrotriazines on the growth of *S. pyogenes*, in terms of fractional inhibitory concentrations, for: (a), trypticase-soy broth (TSB) medium; (b), enzymed casein–casamino acids medium; (c), amino acid medium; (d), amino acid medium supplemented with 10% horse serum.

by Elion *et al.*[6], from which the sum of the fractional inhibitory concentrations of the most effective combinations of the two inhibitors are determined. This value was 1 for TSB medium, indicating no synergism as was reported by Fisher and Doub[1]; whereas it was 0·15 for the enzymed casein–casamino acids medium and 0·066 for the amino acid medium, indicating very good synergism with these two media, especially the

latter. In part (d) of Fig. 1 are shown the data for the amino acid medium to which 10% horse serum had been added. The value here was 0·15, indicating a high degree of synergism still remaining.

The most effective agent found for reversing the synergism of triazine and sulphonamide was DNA, as indicated in Table 5, in which it is shown that a culture of *S.*

TABLE 5. REVERSAL OF TRIAZINE–SULPHONAMIDE SYNERGISM IN AMINO ACID MEDIUM CONTAINING 2 mg SODIUM ACETATE, 0·02 μg TRIAZINE AND 1 μg SULPHONAMIDE PER ml

Substances added per ml	Hours incubation		
	20	40	96
	Turbidity reading (% transmission)		
None	95	95	95
200 μg DNA	25	25	25
40 μg deoxyguanylic acid	95	95	95
40 μg thymidylic acid	95	95	95
40 μg each of deoxyguanylic acid and thymidylic acid	70	30	25

pyogenes remained inhibited for as long as 96 hr in the presence of a combination of these two inhibitors, while DNA caused complete reversal after only a 20 hr incubation. Whereas neither deoxyguanylic acid nor thymidylic acid was effective alone in reversing this synergism, a combination of these two deoxyribonucleotides was active after a 40 hr incubation. This was the only combination of the hydrolytic products of DNA showing activity. The failure of PABA to reverse the synergism is indicated in Table 6. Only 0·02 μg of PABA was required to reverse 10 μg of sulphonamide alone,

TABLE 6. INEFFECTIVENESS OF PABA IN REVERSING TRIAZINE–SULPHONAMIDE SYNERGISM IN AMINO ACID MEDIUM

Triazine (μg/ml)	Sulphonamide (μg/ml)	PABA for complete reversal (μg/ml)
0	10	0·02
0·001	10	0·1
0·01	10	20
0·02	2	>20

whereas 20 μg of PABA was insufficient to reverse the inhibitory effect of 2 μg of sulphonamide in the presence of 0·02 μg of triazine.

Factors affecting the development of tolerance to triazine

We have used the term reversal to describe the overcoming of the inhibitory action of triazines which occurs in a growing culture during an incubation period of from 20 to 40 hr. The development of tolerance, which occurs slowly and only after a number of transfers, was also studied by transferring cultures of *S. pyogenes* daily in the amino acid medium containing no thiamine, and in the same medium with various supplements. After each transfer the amount of triazine needed to produce half-inhibition of growth was determined. Table 7 shows the behaviour of the culture

TABLE 7. DEVELOPMENT OF TOLERANCE TO TRIAZINE ON REPEATED TRANSFERS IN AMINO ACID MEDIUM

Substances added per ml	Times transferred					
	0	1	2	3	4	5
	Triazine for half-inhibition (μg/ml)					
None	0·02	0·01	0·02	0·02	0·02	0·01
1 mg acetate	0·02	0·01	0·02	0·02	0·02	0·01
1 mg acetate + 0·1 μg thiamine	0·02	0·01	0·02	0·03	0·03	0·01
20 μg guanine	0·02	0·01	0·02	0·02	0·02	0·01
20 μg guanine + 0·1 μg thiamine	0·02	0·01	0·01	0·01	0·04	0·05
1 mg acetate + 20 μg guanine	0·05	0·05	0·10	0·20	0·30	0·30
1 mg acetate + 20 μg guanine + 0·1 μg thiamine	0·10	0·08	0·20	2·00	5·00	50·0

after five transfers in the various amino acid media. There was no increased tolerance to triazine in the amino acid medium alone, nor when it was supplemented with acetate or guanine, or acetate plus thiamine, or guanine plus thiamine. There was some increased tolerance to triazine, beginning with the second transfer, when both acetate and guanine were added; but the most rapid and extensive development of tolerance was in the medium in which acetate, guanine and thiamine were all present. However, as shown in Table 8, the stimulating effect of acetate, guanine and thiamine on the

TABLE 8. BLOCKADE OF TOLERANCE TO TRIAZINE BY SULPHONAMIDES IN AMINO ACID MEDIUM

Substances added per ml	Times transferred					
	0	1	2	3	4	5
	Triazine for half-inhibition (μg/ml)					
2 μg sulphonamide	0·002	0·002	0·001	0·001	0·001	0·001
2 μg sulphonamide + 1 mg acetate 20 μg guanine	0·004	0·004	0·003	0·004	0·006	0·004
2 μg sulphonamide + 1 mg acetate + 20 μg guanine + 0·1 μg thiamine	0·01	0·01	0·02	0·02	0·004	0·004

development of tolerance to triazine was overcome, at least through five transfers, by the addition of a small amount of sulphonamide.

DISCUSSION

In addition to the data for the triazine and sulphonamide reported in the text and tables, similar studies with triazines 2 and 3, and with sulphanilamide and sulphamethoxypyridazine, were carried out with similar results. The inhibition of *S. pyogenes* by triazines 2 and 3 could not be reversed by PGA or leucovorin; these triazines were also synergistic with the sulphonamides; and the behaviour of these triazines and sulphonamides corresponded qualitatively in every way tested with the behaviour of the triazine and mixture of sulphonamides reported above.

The fact that reversal of triazines by acetate, in a minimal defined medium containing added DNA, was competitive would imply utilization of the acetate to form a succeeding metabolic product, rather than its substitution for a preceding metabolic product. We were unable to demonstrate any activity for DL-thioctic acid in reversing triazines under our conditions. Broquist and Stiffey[7] recently reported that an analogue of thioctic acid, 6-ethyl-8-mercaptanoic acid, inhibited *Torula cremoris* and that this inhibition was reversed competitively by acetate. Tween 40 (polyoxyethylene sorbitan monopalmitate) and Tween 80 (polyoxyethylene sorbitan mono-oleate) also reversed this inhibition, which the authors interpreted as indicating that acetate was probably being converted into long chain fatty acids. However, we found that neither Tween 40 nor Tween 80 would reverse the triazines under conditions in which acetate was active; nor was malate, citrate, succinate or mevalonate active.

We were led to suspect that thiamine might have a role, either in reversing triazine inhibition of *S. pyogenes* or in the development of tolerance to triazines, in this organism by our previous finding[2] that thiamine reversed the inhibitory action of triazines against *Lactobacillus arabinosus* growing in a minimal medium. The difference in response of these two organisms to thiamine, in a medium containing triazine, was that *L. arabinosus* grew out rather quickly while *S. pyogenes* required several successive transfers. Furthermore, these studies reveal certain other differences between the reversal of triazines in *S. pyogenes* and in other organisms. Leucovorin reverses the triazine inhibition of *Streptococcus faecalis*, and to a certain extent that of *L. arabinosus*,[2] but no reversal could be achieved by this compound in *S. pyogenes*. Combinations of purines and thymidine readily reverse the action of triazines on *S. faecalis* and *L. arabinosus*, while the triazine inhibition of *S. pyogenes* is not easily reversed by these combinations. DNA was the most effective triazine-reversal agent in *S. pyogenes* and the closer the combinations of hydrolytic products from DNA came to the parent compound, the better reversing agents they were. Acetate has not been shown to play a part in the reversal of triazine inhibition in the above-mentioned organisms, yet in *S. pyogenes* it is essential.

Since neither PABA, PGA nor CF reverses the synergism achieved between triazines and sulphonamides in the inhibition of *S. pyogenes*, this synergism would appear not to be effected by sequential blocking along the pathway leading from PABA through folic acid-derived coenzymes to the biosynthesis of components of nucleic acid, such as might be expected from simply combining a sulphonamide, an antagonist of PABA, and a triazine, a recognized antagonist of PGA or CF in other micro-organisms.

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THE EFFECT OF CHLORPROMAZINE HYDROCHLORIDE ON GLUTAMIC ACID DECARBOXYLASE AND PYRIDOXAL KINASE IN RAT BRAIN HOMOGENATES

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Abstract—The effect of the *in vivo* administration of chlorpromazine hydrochloride on glutamic acid decarboxylase and pyridoxal kinase activity was investigated in brain homogenates from normal and vitamin B_6-deficient rats. Chlorpromazine had little or no direct effect on rat brain glutamic acid decarboxylase activity, but was found to be a potent inhibitor of rat brain pyridoxal kinase activity. Pyridoxal kinase in brain homogenates from vitamin B_6-deficient rats showed greater susceptibility to inhibition by chlorpromazine than did pyridoxal kinase from rats on a complete diet.

INTRODUCTION

A DEFICIENCY of vitamin B_6 has been shown to cause central nervous system disturbances in both man[1] and animals.[2] The enzymes responsible for the production of many neurohumours (serotonin, γ-aminobutyric acid, histamine, epinephrine and norepinephrine) are vitamin B_6-dependent.[3–5]

A systematic study of the effect of the neurotropic drugs on some of these enzymes has included an investigation of the effect of chlorpromazine hydrochloride (Cpz) on the activity of glutamic acid decarboxylase (GAD) and on the activity of pyridoxal kinase (Pal-K). Glutamic acid decarboxylase is dependent upon Pal-K for its coenzyme, pyridoxal-5-phosphate (Pal-5-P).[6–10]

EXPERIMENTAL

Female, Long–Evans strain rats were used throughout the experiment. The animals were raised and maintained from weaning either on diets deficient in vitamin B_6 or on diets supplemented with the vitamin. Each of these two dietary groups was divided into three subgroups and received: (a) no drug supplement; (b) 400 mg Cpz per kg diet from weaning; or (c) 25 mg Cpz per kg body weight administered subcutaneously daily for approximately 3 weeks prior to sacrifice. All rats were on their respective diets for from 15 to 18 weeks prior to sacrifice. Vitamin B_6-deficiency was established by the fact that the deficient rats grew poorly and exhibited alopecia. The average daily consumption of Cpz at the time of sacrifice was 28 mg/kg body wt per day or 2·5 mg/rat per day for the vitamin B_6-deficient rats. For rats supplemented with vitamin B_6, the consumption of Cpz was 19·6 mg/kg body wt per day or 4·1 mg/rat per day. All foods, with or without the drug, were supplied *ad libitum* to the time of sacrifice by decapitation; the animals injected with Cpz were sacrificed approximately 20 hr after the last dose of the drug.

The brains were removed intact, washed in buffer, weighed and homogenized in a Potter–Elvehjem homogenizer in 0·05 M KH_2PO_4 (pH 5·0). Evolution of CO_2 at 37 °C was followed in a Warburg apparatus for 70 min; each flask contained 230 mg wet weight of brain as homogenate. To assay for GAD activity the medium contained (final concentration): 0·05 M KH_2PO_4 (pH 5·0), 0·04 M NaF, 0·065 M glutamate and 0·0004 M Pal-5-P. To assay for Pal-K activity, the above medium was modified by omitting Pal-5-P, and by adding 0·016 M ATP, with or without 0·006 M pyridoxal.

DISCUSSION OF RESULTS

Glutamic acid decarboxylase

The effect of dietary Cpz (400 mg/kg diet) on GAD was evaluated in brain homogenates from rats receiving either a complete diet or a vitamin B_6-deficient diet. The effect of Cpz on rat brain GAD is presented in Table 1. The data concerning the

TABLE 1. THE EFFECT OF CHLORPROMAZINE HCl ADMINISTERED *in vivo* ON THE ACTIVITY OF GLUTAMIC ACID DECARBOXYLASE IN RAT BRAIN HOMOGENATES

Group no.	No. of rats	Chlorpromazine HCl administered per kg diet		CO_2(μl) per gramme wet weight in 70 min* Additions to homogenates: 0·065 M Glutamic acid	0·065 M Glutamic acid 0·0004 M Pal-5-P
		Normal diet (plus vitamin B_6)			
(I) Control	10	None		294·0 ± 76·6	552·8 ± 96·5
(II)	5	400 mg	(II) vs. (I)	245·2 ± 34·9 ($P<0·2$)	554·7 ± 68·9 ($P<0·5$)
		Deficient diet (vitamin B_6 omitted)			
(III) Control	9	None		137·5 ± 32·1	718·3 ± 153·4
(IV)	2	400 mg	(IV) vs. (III)	106·0 ± 39·6 ($P>0·5$)	692·2 ± 66·1 ($P>0·5$)

*Expressed as mean values ± standard deviations.

effect of vitamin B_6-deficiency on the GAD activity of untreated animals are in substantial agreement with the findings of Roberts *et al.*[11] The mean values for decarboxylase activity of both groups of rats, on a complete or vitamin B_6-deficient diet and given Cpz, were only slightly lower than the mean values for the respective control groups, indicating that Cpz had little or no direct effect on this enzyme.

Pyridoxal kinase

The activity of GAD is dependent upon the amount of Pal-5-P available, provided that the enzyme is not saturated with the cofactor. The substantial increase in the amount of CO_2 evolved, upon the addition of Pal-5-P to the incubation media of the brain homogenates, is evidence that GAD in these brain homogenates was not saturated with Pal-5-P (see Table 1).[11] Any increase in CO_2 production by the homogenates incubated with ATP and pyridoxal, as compared with homogenates incubated with ATP but without pyridoxal, was considered to be proportional to the amount of Pal-5-P synthesized.

The addition of pyridoxal and ATP to homogenates from normal control rats on a complete diet increased the CO_2 production by 109 μl (Table 2), as compared with the

Table 2. Effect of chlorpromazine HCl (administered orally, 400 mg/kg diet) on the synthesis of pyridoxal-5-phosphate in rat brain homogenates incubated with ATP and pyridoxal (Pal)*

Group no.	No. of rats	CpzHCl admin. per kg diet (mg)	CO_2(μl) formed per g in 70 min Additions to homogenates: 0·065 M Glut. acid 0·016 M ATP (a)	0·065 M Glut. acid 0·016 M ATP 0·006 M Pal (b)	Pyridoxal kinase activity† (c)
			Complete diet (plus vitamin B_6)		
(V) Control	4	None	306·5 ± 49·7	415·8 ± 100·2	109·3 ± 46·4
(VI)	2	400	356·5 ± 17·7	394·5 ± 13·5	38·0 ± 31·1 [0·1 < *P*(VI:V) < 0·2]
			Deficient diet (vitamin B_6 omitted)		
(VII)	4	None	168·0 ± 16·0	501·8 ± 59·6	333·8 ± 97·3
(VIII) Control	2	400	112·5 ± 15·0	131·0 ± 46·7	18·5 ± 2·1 [0·01 < *P*(VIII:VII) < 0·02]

*Expressed as mean values ± standard deviations.
†Net increase in CO_2 formation (col. (b) − col. (a)).

corresponding control homogenates incubated without added pyridoxal. When pyridoxal and ATP were added to brain homogenates from rats on a complete diet containing 400 mg Cpz per kg, CO_2 production was increased by only 38 μl (Table 2), as compared with homogenates containing no pyridoxal. This decrease in activation of GAD by pyridoxal and ATP in brain homogenates from Cpz-treated rats indicated a 65 per cent inhibition of phosphorylation of pyridoxal.

Similar results were obtained with rats receiving 25 mg Cpz per day by subcutaneous injection for approximately 3 weeks prior to the study. The addition of pyridoxal, as well as ATP, to the media of brain homogenates from these animals increased CO_2 production by only 44 μl (Table 3), as compared to a control value of 109 μl indicating a 60 per cent inhibition of Pal-K activity by the drug.

Pal-K activity in brain homogenates from vitamin B_6-deficient rats was even more susceptible to Cpz inhibition. Upon the addition of pyridoxal to brain homogenates from untreated, vitamin B_6-deficient rats, CO_2 production was increased by 334 μl (Table 2), while the increase in CO_2 production upon the addition of pyridoxal to brain homogenates from vitamin B_6-deficient rats receiving 400 mg Cpz/kg diet was only 18 μl, indicating a 95 per cent inhibition of brain Pal-K activity. When Cpz was administered to vitamin B_6-deficient rats by subcutaneous injection (25 mg/kg body wt), the CO_2 production of the brain homogenates containing added pyridoxal

increased by only 36 μl (Table 3), as compared to the homogenates containing no added pyridoxal. This finding indicates that in the deficient rats 89 per cent of the brain Pal-K activity was inhibited by the injected Cpz.

TABLE 3. EFFECT OF CHLORPROMAZINE HCl (SUBCUT. INJECTION, 25 mg/kg per day FOR 2–3 WEEKS) ON THE SYNTHESIS OF PYRIDOXAL-5-PHOSPHATE IN RAT BRAIN HOMOGENATES INCUBATED WITH ATP AND PYRIDOXAL (Pal)*

Group no.	No. of rats	CpzHCl inject. per kg body wt (mg)	CO_2 formed (μl) per g in 70 min Additions to homogenates		Pyridoxal kinase activity†
			0·065 M Glut. acid 0·016 M ATP (a)	0·065 M Glut. acid 0·016 M ATP 0·006 M Pal (b)	(c)
			Complete diet (plus vitamin B_6)		
(V) Control	(From Table 2)	None	306·5 ± 49·7	415·8 ± 100·2	109·3 ± 46·4
(IX)	4	25	242·0 ± 44·7	285·8 ± 69·5	43·8 ± 32·7 [0·02 < P(IX:V) < 0·05]
			Deficient diet (vitamin B_6 omitted)		
(VII) Control	(From Table 2)	None	168·0 ± 16·0	501·8 ± 59·6	333·8 ± 97·3
(X)	2	25	123·5 ± 4·9	159·5 ± 4·9	36·0 ± 7·0 [0·02 < P(X:VII) < 0·05]

*Expressed as mean values ± standard deviations.
†Net increase in CO_2 formation (col. (b) − col. (a)).

In these experiments, the administration of Cpz to the intact animals ensured that the drug levels present in the homogenates were realistic from a pharmacological standpoint. However, the magnitude of the kinase inhibition observed may not necessarily be a reflection of the status of brain Pal-K activity *in vivo*, since homogenization of the brain might have enhanced the inhibitory action of Cpz by disrupting the organization of enzymes *in situ*, thereby increasing their accessibility to the drug. Nevertheless, this experiment indicates that under these conditions Cpz is a potent inhibitor of Pal-K activity. In any attempt to relate the pharmacological action of the drug to the effect of Cpz on Pal-K, other factors need to be taken into consideration, such as the effect of Pal-K on the activity of other vitamin B_6-dependent enzymes, which in turn may affect the levels of other neurohumours.

SUMMARY

Although vitamin B_6-deficiency decreases glutamic acid decarboxylase activity in the brain of rats, chlorpromazine HCl administered *in vivo* appears to have little or no direct effect on this enzyme in either normal or vitamin B_6-deficient rats.

By utilizing the pyridoxal-5-phosphate activation of glutamic acid decarboxylase

as an assay for pyridoxal kinase activity, we found that chlorpromazine HCl had a significant inhibitory effect on pyridoxal kinase activity in homogenates of brains from normal and vitamin B_6-deficient rats.

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SHORT COMMUNICATIONS

The possible biochemical lesion in blindness due to methanol poisoning

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IT is now generally agreed that the ocular toxicity in methanol poisoning is mediated by its metabolic derivative, formaldehyde, that the retina is the primary site of attack in the eye, and that only primates exhibit this peculiar toxicity.[1] The dose of methanol in man which results in ocular changes is not known with any accuracy but the ingestion of 100 ml of methanol is assumed by most investigators to be fatal.[2] Assuming an even distribution of the alcohol in the body water, it may be estimated that in a 70 kg man this dose would result in a concentration of approximately 0·07 M in the body tissues.

Since the retina has the highest aerobic glycolytic rate of any tissue,[3] and since it utilizes glucose primarily if not solely as its source of energy, a likely place to look for a formaldehyde effect would be on the glycolytic enzymes of the retina. Similarly, if a glycolytic enzyme were inhibited by a pharmacological concentration of formaldehyde then it might follow that a deprivation of adenosine triphosphate (ATP) to the retinal cells, a situation analogous to anoxia, even though the inhibition might be temporary, could cause degeneration of those retinal cells concerned with vision and ultimately lead to blindness.

Accordingly, retinal homogenates of rabbit were prepared, incubated with glucose in the presence and absence of 0·005 M formaldehyde and the production of lactic acid was determined. As shown in Table 1, the formation of lactic acid was reduced to one-half that obtained in the absence of the

TABLE 1. INHIBITION OF RETINAL HEXOKINASE BY FORMALDEHYDE

Substrate	Inhibitor	Lactic acid formed (μmoles)	Inhibition (%)
Glucose	None	14·7	—
Glucose	Formaldehyde (0·005 M)	6·3	57
Glucose	Formaldehyde (0·01 M)	2·3	84
Glucose	Methanol (0·005 M)	14·8	0
Fructose–1 : 6–diphosphate	None	6·1	—
Fructose–1 : 6–diphosphate	Formaldehyde (0.005 M)	6·1	0
Fructose–1 : 6–diphosphate	Formaldehyde (0·01 M)	6·3	0
Glucose–6–phosphate	None	10·7	—
Glucose–6–phosphate	Formaldehyde (0·005 M)	10·7	0
Glucose–6–phosphate	Formaldehyde (0·01 M)	10·7	0

Each test tube contained 24 μmoles of substrate, 20 μmoles of potassium phosphate buffer (pH 7·5), 35 μmoles of nicotinamide, 1·5 μmoles of DPN, 1 μmole of ATP, 7 μmoles of $MgCl_2$, 16 μmoles of $KHCO_3$, 0·3 ml of beef retinal homogenate (1:2 with water) and water to a final volume of 2 ml. Incubation was carried out in air for 1 hr at 37°. Lactic acid was determined on the deproteinized filtrates by the method of Barker and Summerson[8].

inhibitor. If, instead of glucose, hexose diphosphate was used as a substrate, formaldehyde caused no inhibition. This would imply that the formaldehyde-sensitive enzyme could be hexokinase, phosphoglucose isomerase or phosphofructokinase or perhaps a combination of these enzymes.* The decisive experiment in which glucose-6-phosphate was employed as a substrate instead of glucose was then performed, and, again, inhibition of lactic acid formation by formaldehyde was not obtained. The

* Potts and Johnson[7] have obtained these results but did not describe any further experiments on the specific site of attack.

results of these experiments are shown in Table 1. Thus, hexokinase is implicated as the primary retinal glycolytic enzyme affected by formaldehyde. These results have been confirmed by direct assays of hexokinase activity in retinal extracts obtained from monkeys, rabbits and cattle.

In an attempt to correlate these findings *in vitro* with those events which occur in methanol poisoning *in vivo*, the vitreous humor of one eye of a rabbit was injected with 0·1 ml of 1 M formaldehyde (the concentration produced in the eye would approximate 0·05 M). The other eye was injected with 0·1 ml of water. A second rabbit was similarly injected in one eye with 0·1 ml of 1 M N-acetylglucosamine,[†] a known competitive inhibitor of hexokinase.[4] Within 1 hr after the injections, the formaldehyde-injected eye on ophthalmological examination appeared greyish-yellow and with a complete lack of pigment design suggestive of an oedematous state, while the untreated eye appeared entirely normal and exhibited a well-defined "pepper-and-salt" fundus with clear retinal vessels. At this time, both eyes of the second rabbit appeared normal, although a suggestion of blurred pigment streaks in the N-acetylglucosamine-treated eye was noted. However, after 24 hr this second rabbit, which had been injected with N-acetylglucosamine, presented the same picture as that described in the eye of the animal injected with formaldehyde; the pigment in the injected eye was cloudy and the retinal vessels were oedematous, while the control eye had a normal appearance. Within 3 days after the start of the experiment, the injected eyes of both rabbits were completely opaque to ophthalmological examination while the control eyes were clear. At 7 days no further change was noted. The animals were then, sacrificed, the retinas removed, homogenized and incubated with glucose. Normal glycolytic activity was observed in the control eyes and in the N-acetylglucosamine-treated eye, but essentially no activity was found in the retinal preparation obtained from the formaldehyde-treated eye.[*] In subsequent experiments, in which the concentration of formaldehyde was reduced to one-fifth of that described above, the same blurred pigment streaks were noted in the treated eye, but opacity failed to develop and, on subsequent assay, the glycolytic activity of the retina of this formaldehyde-injected eye was found to be perfectly normal. This surprising observation was clarified by further experiments *in vitro* in which it was demonstrated that the inhibition of hexokinase by formaldehyde was of a competitive type and could be prevented by the addition of excess glucose. These results are shown in Table 2. Thus, although N-acetylglucosamine and formaldehyde are competitive inhibitors of hexoki-

TABLE 2. COMPETITIVE INHIBITION OF HEXOKINASE BY FORMALDEHYDE

Additions	Glucose metabolized (μmoles)
None	0·41
Formaldehyde (0·01 M)	0·03
Glucose (0·01 M) + Formaldehyde (0·01 M)	0·46

Each test tube contained 2 μmoles of glucose, 10 μmoles of $MgCl_2$, 5 μmoles of ATP, 50 μmoles of tris-buffer (pH 7·4), 0·4 ml of beef retinal homogenate (prepared according to Hoare and Kerly[5]) and water to a final volume of 1·0 ml. Incubation was carried out in air at 30° for 10 min. Glucose was determined on the deproteinized filtrate by the method of Nelson[6].

nase, it would appear that formaldehyde, when present in relatively large amounts, also enters into other reactions possibly of a non-competitive nature.

It is suggested that N-acetylglucosamine or small amounts of formaldehyde inhibit retinal hexokinase for a sufficiently long period to cause retinal degeneration which may lead to blindness. However, because of the metabolism or elimination of these agents and the continual supply of glucose to the retina by way of the blood, hexokinase inhibition is reversible and eventually is overcome.

This thesis is supported by preliminary experiments in which one rabbit was injected in one eye with 0·1 ml of 0·2 M formaldehyde and the control eye was injected with water. A second rabbit was

[*] With this concentration of formaldehyde, opacity and a disappearance of glycolytic activity did not always occur; only when the concentration was doubled was this effect invariable.

injected in one eye with a solution 0·1 ml of 0·2 M formaldehyde in 3 M glucose; the control eye was injected with 0·1 ml of 3 M glucose solution. If our postulate is correct, then, the concomitant administration of glucose together with formaldehyde should prevent the inhibition of hexokinase by the toxic agent and ocular damage should be prevented. Ophthalmological examinations carried out for 12 days revealed that the formaldehyde-treated eye of the first rabbit presented the usual picture of blurred pigment streaks, but the eye of the second rabbit which had received both formaldehyde and glucose showed no difference from that of the control eye.

Additional details concerning this work, together with experiments on the metabolic acidosis observed in methanol poisoning, will be described elsewhere.

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* Taken from a thesis to be submitted by V. T. Marchesi in partial fulfillment of the requirements for the degree of Doctor of Medicine, Yale University.

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Statistical evaluation of the relations between physico-chemical properties and local anaesthetic activity

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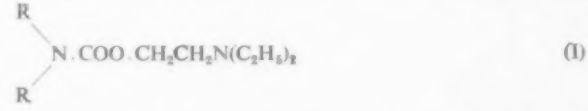
In a basic series of thirty compounds of general formula (I) (R = H, alkyl, phenyl, tolyl, xylyl, alkoxyphenyl, naphtyl),[1] we have studied the relations between the local anaesthetic action and: (a) tensioactivity (Traube's stalagmometer, aqueous 0·05 M solutions non-buffered and buffered pH



(I)

7·0), (b) displacement adsorption (adsorbate: active charcoal/methylene blue), (c) distribution coefficient (ether/phosphate buffer pH 7·4), and (d) ability to coagulate colloids (colloidal solution of mastix). In order to avoid the shortcoming of certain earlier studies, in which the writers compared the exact values of physico-chemical measures with the relative activities of only one kind of anaesthesia, the values obtained have been compared with the relative activities of three kinds of anaesthesia[2, 3, 4]: surface (rabbit cornea, 0·05 M cocaine as standard), infiltration (intraderma 0·05 M appllication to guinea pigs, procaine as standard), and conduction (frog ischiadic nerve, 0·01 M procaine as standard) The relative activities were calculated (method *A*) as the quotients of the duration of the complete anaesthesia after the application of the aqueous equimolar solutions of the substances studied and of the standards utilized.

Later on, according to a more precise technique[5] (method *B*), the evaluation of surface and infiltration anaesthetic activity was based on the determination of the molar concentrations of the compounds studied, which have the same activity as the selected concentrations of the standards. We have then abandoned the study of conduction anaesthesia because this type has similarities with infiltration anaesthesia and because the method used was inexact.

The correlation coefficients of the physico-chemical properties studied with the relative activities measured by the different methods used are shown in Table 1. They are in general higher for method *B*. This results probably from its greater precision. On the other hand this interpretation agrees with the low coefficients found in the case of conduction anaesthesia studied by the inexact method already mentioned.

It is interesting to notice the increase of the correlation coefficients in the series: tensioactivity (non-buffered solutions) < tensioactivity (buffered solutions) < displacement adsorption < ability to coagulate colloids. This may be related to the process of adsorption taking place in the organism and may also indicate that ability to coagulate colloids is a property of importance in the mechanism of local anaesthetic action.

TABLE 1. STATISTICAL EVALUATION OF THE RELATIONS STUDIED
(Spearman's Correlation Coefficient)

Physicochemical property	Relative activity calculated				
	From the duration of the anaesthesia (method *A*)*			From the equieffective concentration (method *B*)	
	Surface	Infiltration	Conduction	Surface	Infiltration
Tensioactivity (non-buffered solutions)	0·745 ± 0·57	0·776 ± 0·052	0·601 ± 0·083	0·759 ± 0·054	0·798 ± 0·047
Tensioactivity (buffered solutions)	0·852 ± 0·035	0·817 ± 0·043	0·716 ± 0·063	0·853 ± 0·035	0·869 ± 0·031
Displacement adsorption	0·880 ± 0·029	0·879 ± 0·029	0·742 ± 0·058	0·865 ± 0·033	0·892 ± 0·026
Distribution coefficient	0·882 ± 0·029	0·878 ± 0·030	0·767 ± 0·053	0·881 ± 0·029	0·914 ± 0·021
Ability to coagulate colloids	0·905 ± 0·023	0·886 ± 0·028	0·817 ± 0·043	0·912 ± 0·022	0·913 ± 0·021

* Values taken from previous papers[2, 4].

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Salicylate and intestinal absorption of glucose in the rat

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Salicylate causes increased blood glucose concentrations in the rat given oral glucose and this was considered to be due to an increased intestinal absorption of the sugar.[1] However, this interpretation is questionable since salicylate produces a hyper-glycaemic response in the fasted rat not receiving glucose[2] and also because the substance has no effect on the absorption of glucose by perfused intestinal loops.[3] The present experiments were designed to study the effect of salicylate on the *in vivo* absorption of glucose in the rat by the method of Cordier and Touzé[4]. Male Wistar rats, 200 to 300 g wt, were fasted for 48 hr and then anaesthetized with ether. The abdomen was opened and ligatures tied at the junction of the pylorus and duodenum and at the bottom end of the ileum. Two ml of isotonic (5·4 per cent w/v) glucose was injected into the portion of gut between the ligatures and about 1 ml of salicylate solution (to give either 350 or 400 mg/kg body weight of salicylate) or 1 ml of 0·9 per cent saline (control animals) injected into an exposed femoral vein. The animals were maintained under ether anaesthesia for either 30 or 60 min, then killed by decapitation, the portion of intestine between the ligatures was removed and its contents were washed out as described by Cordier and Touzé[4]. The washings were treated according to the directions of Hele[5] and the glucose content estimated by the method of Nelson[6]. A specimen of heart blood obtained at the end of the experiment was analysed for salicylates by the method of Trinder[7]. The results given in Table 1 show that salicylate had no effect on either the 30 min or 60 min absorption of glucose by rat small intestine.

Table 1. Effect of salicylate on the intestinal absorption of glucose in the rat

Results are given as means, standard deviations and ranges, the number of animals in each group being stated in parentheses. The results have been analysed by the *t*-test and values of *P* are included.

Animals	Duration of expt. (min)	Blood salicylate (mg/100 ml) Mean ± s.d.	Range	Absorption of glucose (mg) Mean ± s.d.	Range	*P*
Control (10)	30	—	—	56±7	48–68	0·6
Salicylate (8)	30	50±4	43–55	54±8	41–65	
Control (8)	60	—	—	77±15	57–95	0·3
Salicylate (8)	60	62±7	53–77	86±13	68–100	

These results confirm the conclusion of Escribano and Ponz[3] that salicylate has no effect on the intestinal absorption of sugar and suggest that the apparently increased absorption observed in the intact rat must be due to other causes.

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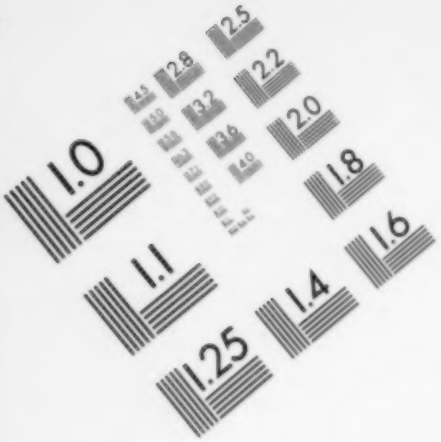

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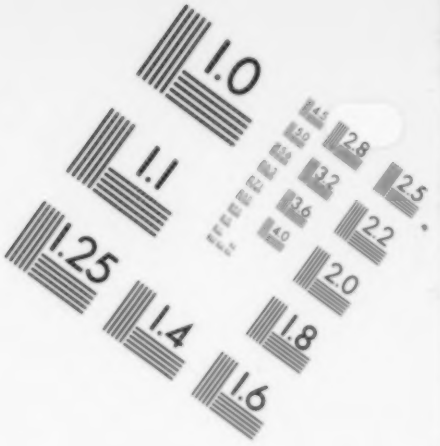

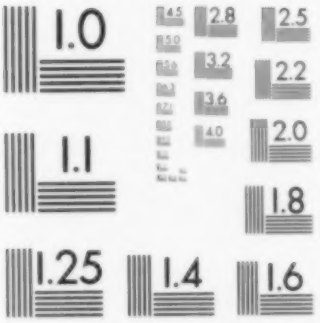

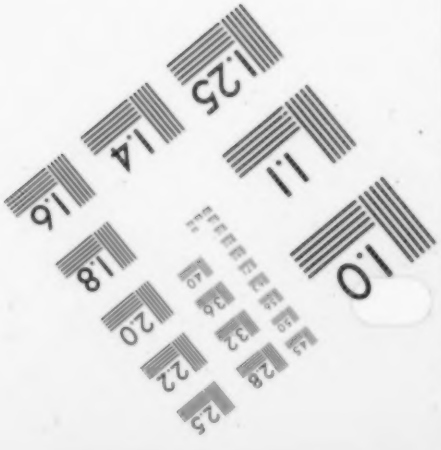

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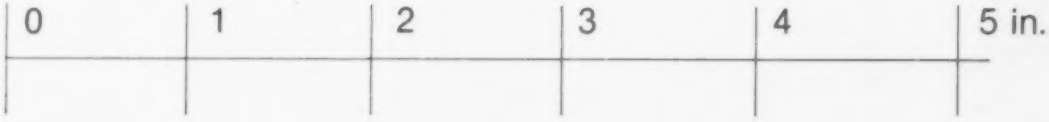

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